Molecular Characterisation and Immunological Analysis of Clinical and Environmental Isolates of *Mycobacterium kansasii* from South African Gold Mines

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of

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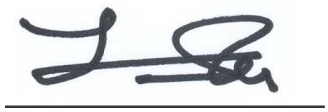
DECLARATION

I, Geoffrey Kwenda, declare that this is my own work. It is being submitted for the Degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree at this or other university.



Geoffrey Kwenda

25th day of June, 2010

DEDICATION

This thesis is dedicated to my mother who taught me that the best kind of knowledge to have is that which is learnt for its own sake.

PUBLICATIONS AND PRESENTATIONS

The following manuscripts, based on this thesis, have been prepared for publication:

- Kwenda, G, Churchyard, G. J, Stevenson, K, Heron, I, Thorrold, C, Duse, A. G and Marais, E (2010). Molecular characterisation of clinical and environmental isolates of *Mycobacterium kansasii* from a South African gold-mining region. (Manuscript I).

- Kwenda, G, Duse, A. G and Marais, E (2010). Genetic differences between clinical and environmental isolates of *Mycobacterium kansasii*. (Manuscript II).

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ABSTRACT

The South African gold-mining workforce has an unusually high incidence of *Mycobacterium kansasii* disease, yet little is known about the possible sources of *M. kansasii* infection, genetic diversity and the basis for this organism's pathogenicity. The purpose of this study was to investigate these issues in a gold-mining environment. Five *M. kansasii* isolates and 10 other potentially pathogenic mycobacteria were cultured mainly from showerhead biofilms. PCR-restriction analysis (PRA) of the *hsp65* gene on 191 clinical and on the 5 environmental *M. kansasii* isolates revealed 160 subtype I (157 clinical and 3 environmental), 8 subtype II (clinical) and 6 subtype IV (5 clinical and 1 environmental) strains. Twenty-two isolates (21 clinical and 1 environmental) did not show the typical *M. kansasii* PRA patterns. After confirmation by DNA sequencing as belonging to the *M. kansasii* species, the results suggested that these isolates were probably new subtypes of *M. kansasii*. In contrast to the clonal population structure found amongst the subtype I isolates from studies in other countries, DNA fingerprinting of 114 subtype I clinical and environmental isolates showed genetic diversity amongst the isolates. One of the environmental isolates showed 100% identity with a clinical isolate, suggesting that water distribution systems are possible sources of *M. kansasii* infection for the miners. An investigation into the genetic differences between clinical (subtype I) and environmental (III, IV and V) isolates, using Hybridisation Monitored Differential Analysis (HMDA), identified 45 open reading frames (ORFs) encoding predominantly membrane-associated proteins that include six potential virulence factors, two family members of transcription regulators for drug and xenobiotic metabolism, three family members of multidrug efflux systems, a number of proteins associated with lipid and carbohydrate metabolism and transport, and a number of hypothetical proteins with unknown function. Immunological analysis of *M. kansasii* isolates, using the Lymphocyte Transformation and Cytometric Bead Array assays, showed that *M. kansasii* modulates immune responses through suppression of lymphocyte blastogenesis and by altering the expression of Th1/Th2/Th17 cytokines by human lymphocytes *in vivo* for its own survival. This study demonstrated for the first time that water distribution systems in South Africa are possible sources of *M. kansasii* infection, and showed that subtype I strains of *M. kansasii* from the study region display genetic diversity and have unique or divergent genes not found in other subtypes. It also demonstrated that immunosuppression is one of the pathogenic mechanisms employed by *M. kansasii*.

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LIST OF ABBREVIATIONS

AFB	Acid-Fast Bacilli
AFLP	Amplified-Fragment Length Polymorphism
AIDS	Acquired Immunodeficiency Syndrome
AraLAM	Non-mannose-capped lipoarabimannan
APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
ATM	Atypical Mycobacteria
ATS	American Thoracic Society
BAL	Broncho-Alveolar Lavage
BCG	Bacille Calmette-Guérin
CBA	Cytometric Bead Array
CXCL	Cysteine-(X=another amino acid)-Cysteine Ligand
CD	Cluster of Differentiation
cDNA	complementary Deoxyribonucleic Acid
CFP-10	Culture Filtrate Protein, 10 kDa
CFU	Colony Forming Units
COG	Cluster of Orthologous Groups of proteins
COPD	Chronic Obstructive Lung Disease
ConA	Concanavalin A
CPC	Cetylpyridinium Chloride
CR	Complement Receptor
CSPD	Sodium 3-{4-meth-oxyspiro[1,2-dioxetane-2,2'-(5'-chloro) tricycle [3.3.1.13,7] decan phenyl phosphate

CTAB	Cetyltrimethylammonium bromide
DAT	2,3-di-O-acyl-trahalose
DC-SIGN	Dendritic Cell-Specific Intracellular Adhesion Molecule-Grabbing Non-integrin
DNA	Deoxyribonucleic Acid
dNTP	deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic Acid
EM	Environmental Mycobacteria
ESAT-6	Early Secreted Antigenic Target, 6 kDa
ESP solution	EDTA-Sarkosyl-Proteinase K solution
ESX-1	ESAT-6 Secretion System 1
FOXP3	Foxhead Box P3
GC	Guanine Cytosine
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte Monocyte-Colony Stimulating Factor
GFE	Genome Fraction Enrichment
GPI	Glycosylphatidylinositol
HIV	Human Immunodeficiency Virus
HMDA	Hybridisation-Monitored Genome Differential Analysis
HPLC	High Performance Liquid Chromatography
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible Nitric Oxide
IPTG	Isopropyl β-D-1-galactopyranoside

ITS	Internal Transcribed Sequence
IWGMT	International Working Group on Mycobacteria Taxonomy
kb	kilobase
kanLAM	*M. kansasii* (mannose-capped) Lipoarabimannan
kanLM	*M. kansasii* (non-mannose-capped) Lipomannan
LAM	Lipoarabimannan
LB	Luria-Bertani culture medium
LJ	Löwenstein-Jensen
LM	Lipomannam
LT	Lymphocyte Transformation
MAC	*Mycobacterium avium* Complex
ManLAM	Mannose-capped Lipoarabimannan
MCE	Mammalian Cell Entry
MHC	Major Histocompatibility Complex
MGIT 960	Mycobacterial Growth Indicator Tube 960
M7H9/10	Middlebrook 7H9/10 culture media
MK	*Mycobacterium kansasii*
MmpL	Mycobacterial Membrane Protein Large
MN cells	Mononuclear cells
MOTT	Mycobacteria Other Than *Mycobacterium tuberculosis*
MR	Mannose Receptor
mRNA	Messenger Ribonucleic Acid
Mtb	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* Complex
MyD88	Myeloid Differentiation Primary Response Gene 88

NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
NF-κB	Nuclear Factor Kappa B
NHLS	National Health Laboratory Service
NK cell	Natural Killer cell
NNRT	Non-Nucleoside Reverse Transcriptase Inhibitor
NO	Nitric Oxide
NTM	Nontuberculous Mycobacteria
NR	Non-Redundant
OADC	Oleic Acid Albumin Dextrose, M7H9/10 media supplement
OD	Optical Density
ORF	Open Reading Frame
PAMPs	Pathogenic-Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PDIM	Phthiocerol dimycocerosate
PE	Proline-Glutamine
PE-PGRS	Proline-Glutamine-Polymorphic GC-Rich Sequences
PFGE	Pulsed-Field Gel Electrophoresis
PGL	Phenolic Glycolipids
PHA	Phytohemagglutinin
PI	Protease Inhibitor
PPE	Proline-Proline-Glutamate
PRA	PCR-Restriction Analysis
PRASITE	PCR-Restriction Analysis Site
ProtClustDB	Protein Clusters Database

RD	Regions of Difference
rDNA gene	Ribosomal Deoxyribonucleic Acid gene
RNA	Ribonucleic Acid
RND	Resistance, Nodulation and Cell Division
rRNA	Ribosomal Ribonucleic Acid
SC	Suppressor Carbohydrates
SCAF	Suppressor Cell Activating Factors
SCOTS	Selective Capture of Transcribed Sequences
SDS	Sodium Dodecyl Sulphate
SI	Similarity Index
SIMRAC	Safety In Mines Research Advisory Committee
SL	Sulpholipids
SOC	Super Optimal Catabolite
SOD	Superoxide Dismutase
SOS	Son of Sevenless
SSC buffer	Saline Sodium Citrate buffer
TBE	Tris-Borate EDTA
TCR	T Cell Receptor
TDM	Trehalose 6,6'-dimycolate
TE	Tris-EDTA
TetR	Tetracycline Resistance
TGF-β	Transforming Growth Factor - beta
Th cells	T helper lymphocytes
TLR	Toll-Like Receptor
TNF-α	Tumour Necrosis Factor - alpha

Tris	Tris(hydroxymethyl)aminomethane
Tween	Polyoxyethylene sorbitan monooleate
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3 indolyl-β-galactoside
XRE	Xenobiotic Response Element
ZN	Ziehl-Neelsen

CHAPTER 1

General Introduction

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1.0 Introduction

1.1 Background

The genus *Mycobacterium* comprises more than 130 species, including pathogens and saprophytes (Gutierrez *et al*, 2009). Based on their clinical significance, these species can be divided into three groups. The first group includes obligate pathogens for humans and animals, i.e, the *Mycobacterium tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. canettii, M. pinnipedii* and *M. caprae*), *M. leprae* and *M. lepraemurium*, which are generally not found in the environment. *M. tuberculosis* complex (MTBC), the causative agent for tuberculosis, is by far the most important of the genus *Mycobacterium* from a clinical perspective (Primm *et al*, 2004). The second group comprises mycobacteria that are potentially pathogenic to humans or animals. The majority of these species have been isolated from various terrestrial and aquatic environments and are capable of causing disease in individuals with predisposing conditions. Examples include *M. kansasii*, *M. avium* and the other members of the so-called "*M. avium* complex" (MAC). The third group consists of saprophytic species that are non-pathogenic or only exceptionally pathogenic, e.g, *M. gordonae*, *M. goodii* and *M. smegmatis*. The second and third group are alternatively referred to as Environmental Mycobacteria (EM), Atypical Mycobacteria (ATM), Mycobacteria Other Than *M. tuberculosis* (MOTT) or Non-Tuberculous Mycobacteria (NTM). However, the latter two terminologies are rather confusing because some members of these groups, e.g. *M. kansasii*, can also cause tuberculosis-like lesions (Griffith, 2002; Smith *et al*, 2003; Shitrit *et al*, 2007).

Recently there has been increasing awareness of NTM disease. This may be attributed to the Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS) epidemic, the high incidence of tuberculosis (TB) and other respiratory diseases, and the greater awareness on the part of clinical and laboratory personnel of the impact of NTM on human health (Horsburgh, 1996, Bloch *et al*, 1998; Cattamanchi *et al*, 2008). Furthermore, NTMs are often involved in nosocomial outbreaks (Phillips and von Reyn, 2001), but there is little or no evidence for person-to-person transmission of these organisms. Although the reservoir of infection in most cases remains unclear, there is a general notion that NTM infections derive mainly from the environment (Engel *et al*, 1980, Chang *et al*, 2002, Vaerewijck *et al*, 2005, Falkinham *et al*, 2008; Narang *et al*, 2009). However, the significance of isolation of these organisms in clinical samples remains unclear since the number of diseases they cause is difficult to assess and no system for notification exists as in the case of *M. tuberculosis*.

In South Africa, the gold-mining workforce has been reported to have an unusually high incidence of *M. kansasii* disease (Corbett *et al*, 2000). This high incidence of disease has been attributed to the susceptibility of the miners to a number of risk factors for NTM disease (Corbett *et al*, 1999a; Corbett *et al*, 1999c; Churchyard, 2000; Corbett *et al*, 2000). However, little is known about its natural reservoir, mode of transmission and pathogenicity. Reports on *M. kansasii* from the South African gold mines have mainly focused on its clinical features (Corbett *et al*, 1999a; Corbett *et al*, 1999b; Churchyard *et al*, 1999; Corbett *et al*, 2000). No laboratory studies on *M. kansasii* have been conducted to complement the clinical findings.

1.2 General Characteristics of *M. kansasii*

M. kansasii is an environmental opportunistic pathogen that causes tuberculosis-like disease and is often associated with immunocompromised persons or those who have had lung disease previously (Bloch *et al*, 1998; Razavi and Cleveland, 2000; Griffith, 2002; Smith *et al*, 2003; Arend *et al*, 2004; Yim *et al*, 2005; Griffith *et al*, 2007). It is considered to be the most virulent NTM species and the second most important NTM species, after *M. avium* isolated from patients with AIDS (Wolinsky, 1992; Bloch *et al*, 1998). Unlike other NTM species, most isolates from clinical specimens are considered to be clinically significant, but the sources of infection are difficult to identify (Bloch *et al*, 1998; Griffith, 2002).

M. kansasii is a slow-growing aerobic, photochromogenic, long, beaded, acid-fast bacillus with bent or folded ends arranged in a haphazard fashion that produces predominantly rough or smooth colonies with irregular edges, which develop intense yellow pigmentation on exposure to light (Jenkins *et al*, 1982; Wayne and Kubica, 1986). It requires 2 to 3 weeks' growth over a temperature range of 32 to 42^{o}C, but grows better at 37^{o}C. The biochemical characteristics that form the basis for its identification include production of catalase, nitrate reductase, pyrazinamidase and its ability to hydrolyse Tween 80 (Jenkins *et al*, 1982; Wayne and Kubica, 1986).

Phylogenetic and molecular analyses have demonstrated that *M. kansasii* is a heterogeneous species, with 7 subtypes to date. Subtype I represents the most common isolate from humans (Ross *et al*, 1992; Alcaide *et al*, 1997; Picardeau *et al*, 1997; Richter *et al*, 1999; Taillard *et al*, 2003; Santin *et al*, 2004). This heterogeneity has raised questions as to whether *M. kansasii* is a single species or a complex (Tortoli, 2003a).

1.3 Taxonomic and Phylogenetic Status

Taxonomically, *M. kansasii* belongs to the genus *Mycobacterium*, which is a single genus within the family *Mycobacteriaceae*, under the order *Actinomycetales,* class *Actinomycetes* and phylum *Actinobacteria* (Wayne and Kubica, 1986). The order *Actinomycetales* includes a diverse group of microorganisms, but mycobacteria and allied taxa are easily distinguished on the basis of their ability to synthesise mycolic acids, acid-alcohol fastness and a high GC content (61-71%) in their genomic deoxyribonucleic acid (DNA). *M. kansasii* has a GC content of 66% (Veyrier *et al*, 2009). The only exception is *M. leprae*, which has a low GC content of 54 to 57% (Wayne and Kubica, 1986; Levy-Frebault and Portaels, 1992). The acid-fastness that these organisms exhibit is attributed to the high lipid content in their cell walls that prevent penetration of dyes, which requires the organism first to be heated to allow the dye to penetrate the bacteria. The subsequent destaining process, with an acid or alcohol, removes the dyes from most other bacteria but mycobacteria retain the dye and are thus referred to as acid-fast bacilli (Wayne and Kubica, 1986; Leão *et al*, 2004).

Over the last few decades, a number of tests and techniques have been developed for identifying, characterising and classifying mycobacteria, leading to the generation of a huge amount of information. Thus, there was a great need for consolidating and standardising this information, especially that pertaining to classification. This led to the formation of the International Working Group on Mycobacterial Taxonomy (IWGMT) in 1967, which played a vital role in harmonising approaches to the taxonomy of mycobacteria (Wayne and Kubica, 1986; Wayne *et al*, 1989). Technologies for analysis of semantides are being developed and improved all the time and this has allowed comparisons to be made between phenotypic and genotypic relationships of mycobacteria.

A variety of approaches have been used to characterise the systematics and taxonomy of mycobacteria. The early classification of NTMs was based on the growth rate, pigmentation and clinical significance (Runyon, 1959). A fundamental taxonomic division was tied to growth rate: members of the mycobacteria were defined as either slow or rapid growers. The rapid growers show visible growth within 7 days and the slow growers require more than 7 days for visible growth (Wayne and Kubica, 1986). By these criteria, the genus was divided into the 4 Runyon groups: Group I, slow-growing photochromogens (bacterial colonies produce pigment only with light, e.g *M. kansasii*, *M. marinum*, *M. simiae*); Group II, slow-growing scotochromogens (bacterial colonies produce pigment even in darkness, e.g., *M. scrofulaceum*, *M. szulgai*, *M. gordonae*); Group III, slow-growing non-photochromogens (bacterial colonies produce weak to no pigment in both darkness and light, e.g., *M. malmoense*, *M. xenopi*, *M. avium-intracellulare*); and Group IV, the rapid growers (bacterial colonies grow within 7 days with little to no pigment, e.g. *M. fortuitum*, *M. chelonae*, *M. abscessus*).

Results from cultural and biochemical tests have been analysed by numerical taxonomic techniques to identify clusters of related mycobacteria (Wayne and Diaz, 1982; Wayne *et al*, 1983; Wayne *et al*, 1991), but these have not provided precise boundaries between some species such as between *M. avium* and *M. intracellulare* (Wayne and Kubica, 1986). More recently, other methods have been used to complement the numerical studies and to confer natural relationships among the mycobacteria. These studies include immunological techniques (Shivannavar *et al*, 1996), comparison of cell wall components (Tsang *et al*, 1983; Ausina *et al*, 1992; Ridell, 1993), comparison of homologous enzyme sequences (Ridell, 1993), DNA-DNA hybridisation (Lévy-Fébaulty *et al*, 1986), plasmid profiles (Meissner and Falkinham, 1986) and restriction endonuclease analyses (Ferdinand

et al, 2004). These too have so far failed to provide a unified and unambiguous classification of the genus.

In recent years there has been an emphasis on the development of molecular tools which have led to the profound modifications in the classification and methods of identification of mycobacteria. These methods, utilising one or several appropriate genes, are gaining importance due to the fact that they yield quick and, in most cases, unequivocal results (Ferdinand *et al*, 2004). These methods allow different laboratories to compare their results. Different phylogenies for the genus have been developed using sequence analysis of the 16S rRNA gene (Rogall *et al*, 1990b; Stahl and Urbance, 1990; Harmsen *et al*, 2002). For instance, the number of genes encoding the 16S rRNA has been used to separate slow and rapid growers, with rapid most growers having two copies of the gene and slow growers only having one (Helguera-Repette *et al*, 2004). The 16S rRNA is an approximately 1500bp sequence encoded by the 16S ribosomal DNA (rDNA). The rDNA gene is a highly conserved gene in which regions common to all bacteria exist while nucleotide variations are concentrated in certain areas. In mycobacterial 16S rDNA, the nucleotide stretches are shared by all members of the genus *Mycobacterium* and also contain the hypervariable regions characterised by species-specific variability (Boddinghaus *et al*, 1990). The 16S rRNA-based genetic investigation of mycobacterial taxonomy and phylogeny focuses on two hypervariable sequences, known as region A and region B, which correspond to the *Escherichia coli* positions around 130 to 210 and 430 to 500, respectively (Böttger, 1996).

However, within the genus *Mycobacterium*, the interspecies similarity is relatively high, from 94.3 to 100% (Rogall *et al*, 1990a). Some species have a very high degree of

similarity or have exactly identical sequences such as *M. kansasii* and *M. gastri*, *M. senegalense* and *M. farcinogenes*, *M. marinum* and *M. ulcerans*, *M. malmoense* and *M. szulgai* and members of the *Mycobacterium tuberculosis* complex (Rogall *et al*, 1990a). This suggests that these organisms are phylogenetically related and might be considered subspecies of each other. The use of the 16S rRNA gene has led to problems related to cross-reactivity when oligonucleotide probes are used and makes the design of probes directed towards a broad panel of all clinically important species difficult (Emler *et al*, 1995; Kox *et al*, 1995). The distinction between two species, whether at species or subspecies level, is very important since, for instance, *M. kansasii* is usually significant when isolated from a clinical specimen, whereas *M. gastri* is rarely significant (Turenne *et al*, 2001).

In view of the foregoing problems, a study of more variable sequences in the RNA operon of phylogenetically and closely related species was sought. The genes coding for rRNA are arranged in the order 5'-16S-23S-5S-3' and are separated by two non-coding spacer regions (Klappenbach *et al*, 2000; van der Giessen *et al*, 1994). The 16S-23S rDNA internal transcribed spacer (ITS) has been suggested to represent a potential target within the bacterial genome to find suitable sites for probes and from which to derive additional phylogenic information. This locus is flanked by well-conserved regions of the rRNA operon, containing both conserved and highly variable sequences (van der Giessen *et al*, 1994). The 16S-23S ITS region is considered a useful genomic marker for the subtyping of *M. kansasii* strains (Roth *et al*, 2000; Iwamoto and Saito, 2006).

Apart from the rRNA gene sequences, other genes have been targeted for the identification or classification of mycobacteria. These include *hsp65* (Telenti *et al*, 1993; Ringuet *et al*,

1999; Brunello *et al*, 2001), *sodA* (Zolg and Philippi-Schulz, 1994; Gingeras *et al*, 1998), *rpoB* (Gingeras *et al*, 1998; Kim *et al*, 1999), *recA* (Blackwood *et al*, 2000), *gyrA* (Sander *et al*, 1998), *gyrB* (Kasai *et al*, 2000) and *secA1* (Zelazny *et al*, 2005). The *hsp65* gene is a member of the family of heat-shock protein genes that is highly conserved amongst mycobacterial species. It also presents hypervariable regions (positions 624 to 664 and 683 to 725 of the *M. tuberculosis* gene), whose sequences are used for identification purposes (Ringuet *et al*, 1999). The gene products are highly immunogenic, with an exceptional degree of evolutionary conservation. They function in intracellular protein folding, assembly and transport and their expression is up-regulated under cellular stress. The *sodA* gene encodes superoxide dismutase (SOD), a metalloenzyme that constitutes one of the major defence mechanisms against oxidative stress (Zhang *et al*, 1991; Kang *et al*, 2006) and may play a role in the development of protective immunity against mycobacteria as it has been shown to possess immunogenic properties (Pal and Horwitz, 1992; Orme *et al*, 1993). SOD belongs to a family of proteins which has been conserved throughout evolution, from bacteria to humans (Zhang *et al*, 1995). The *rpoB* gene encodes for the β-subunit of RNA polymerase, an oligomeric enzyme responsible for RNA synthesis (Lesley *et al*, 1987) and its mutation is associated with drug resistance to rifampicin (Klein *et al*, 2001). The product of the *recA* gene plays an essential role in genetic recombination, repair of stalled replication forks, double strand break repairs, induction of SOS response and SOS mutagenesis (Cox, 2003). The *gyrA* and *gyrB* genes code for type II DNA topoisomerases, termed DNA gyrases, responsible for controlling supercoiling of DNA in cells. The A subunit of DNA gyrase, GyrA, is well conserved amongst bacteria and is responsible for mediating double-strand breakage and reunion of DNA, while the other subunit, GyrB, is responsible for energy transduction through ATP hydrolysis (Wigley, 1995). The *secA1* gene is a house-keeping gene that encodes the essential protein, SecA1,

a key component of the major pathway of protein secretion across the cytoplasmic membrane of mycobacterial and other bacterial species (Mori and Ito, 2001).

The use of a single gene for the identification and classification of mycobacteria does not show good discrimination of the bacteria (Kim *et al*, 1999; Stackebrandt *et al*, 2002). In order to increase the discriminatory power of these molecular methods in the identification and phylogenetic studies of bacteria, the combined use of several gene sequences has been recommended (Stackebrandt *et al*, 2002). In addition to the 16S rRNA gene, four to five house-keeping genes are recommended, especially for the description of novel bacterial species. Devulder and colleagues developed a multigene sequence database dedicated to the identification of species within the genus *Mycobacterium* by targeting four genes, *16S rRNA*, *hsp65*, *rpoB* and *sod* (Devulder *et al*, 2005). In this study, they were able to show that the studied genes, which are located in different parts of the genome, evolved in the same manner, and that the use of several genes makes it possible to refine the phylogenetic approach and provides the molecular basis for accurate species identification. Recently, this multigene approach has been used to test the discriminatory power and robustness of the *hsp65*, *16S-23S* ITS, *gryB* and 16S rRNA genes in the phylogenetic analysis of *M. kansasii* strains. The results revealed that an intermediate *M. kansasii* type I strain exists, and suggested that type I strains evolved from type II strains and that there is a possible evolutionary link between the two strains (Iwamoto and Saito, 2006). Type I strains have been mainly isolated from humans, while type II strains have been isolated from both humans and the environment, and the other subtypes have been isolated mainly from the environment (Tortoli, 2003a).

1.4 Epidemiology

The epidemiology of *M. kansasii* is predominantly urban and has been associated with high density and low income communities, especially in highly industrialised areas (Penny *et al*, 1982; Chobot *et al*, 1997; Iinuma *et al*, 1997; Bloch *et al*, 1998; Churchyard, 2000; Santin *et al*, 2004). The organism has been isolated from water distribution systems in the same communities where patients with *M. kansasii* disease have been identified (Steadham, 1980; Chobot *et al*, 1997). Large regional differences have been detected in the incidence of *M. kansasii* infection. Geographical areas reported to have high incidences of *M. kansasii* disease include the Czech Republic (Kubin *et al*, 1980), the United Kingdom (Lamden *et al*, 1996), South Africa (Churchyard, 2000), Spain (Santin *et al*, 2004) and Central and Southern United States (Steadham, 1980; Zhang *et al*, 2004) where it is the major cause of NTM pulmonary disease. There is no evidence for human-to-human transmission of this organism. Although the reservoir of infection in most cases remains unclear, there is a general notion that *M. kansasii* infections may be acquired from the environment, but a definitive link between the environment and human disease has not yet been established. Drinking water distribution systems, tap water and shower heads have been reported to be the major sources of *M. kansasii* (Engel *et al*, 1980; Picardeau *et al*, 1997). Infection probably occurs via an aerosol route (Griffith, 2002).

Recognised risk factors for *M. kansasii* infection include male sex, older age, smoking, malignancy, alcoholism, diabetes, previous mycobacterial disease, chronic obstructive lung disease (COPD), immunosuppression and pre-existing lung disease such as silicosis (Wolinsky, 1992; Bloch *et al*, 1998; Corbett *et al*, 1999c; Corbett *et al*, 2000; Jacobson *et al*, 2000). In South African gold miners, there is an unusually high incidence of *M. kansasii* infection and this has been estimated to be about 320 per 100 000 (Corbett *et al*,

2000). These miners have several risk factors for NTM disease, including HIV infection, high incidence of tuberculosis, silicosis, the extensive use of aerosolised water for dust control by the gold mines and an unusually high occupational exposure to NTM organisms (Corbett *et al*, 1999c; Corbett *et al*, 2000; Churchyard and Corbett, 2001). In Spain and the United Kingdom reported rates were 73 per 100 000 (Pintado *et al*, 1999) and 92 per 100 000 (Klein *et al*, 1998), respectively, while in the United States the rates ranged from 100 to 2667 per 100 000 (Carpenter and Parks, 1991; Bamberger *et al*, 1994; Witzig *et al*, 1995; Bloch *et al*, 1998).

1.5 Comparative Genomics

Advances in molecular biology have revolutionised the field of mycobacterial research. They are providing us with valuable insights into the relationships between genotype and phenotype in mycobacteria and have paved a way for the development of the field of genomics, which refers to the study of all genes that are present in a genome (Brosch *et al*, 2001).

Comparative genomics is the analysis and comparison of genomes from different species. Its purpose is to gain a better understanding of how species have evolved and to determine the function of genes and non-coding regions of the genome. It involves the use of computer programs that can align multiple genomes and look for regions of similarity among them (Brosch *et al*, 2001). The increasing availability of complete bacterial genome sequences is leading to the widespread use of comparative and functional genomics methods for the better understanding of metabolism, identification of unique virulence factors (Brosch *et al*, 2001; Gordon *et al*, 2002), diagnostic and therapeutic

targets (Fitzgerald and Musser, 2001; Randhawa and Bishai, 2002), and evolution of mycobacteria (Brosch *et al*, 2001; Kato-Maeda *et al*, 2001; Cole, 2002; Behr, 2008).

One of the first applications of comparative mycobacterial genomics involved the comparison of macrorestriction profiles using low frequency restriction enzymes and Pulsed-Field Gel Electrophoresis (PFGE). These studies revealed differences amongst the mycobacterial isolates studied (Zhang *et al*, 1995; Iinuma *et al*, 1997; Legrand *et al*, 2001) and contributed significantly to the construction of physical maps that were essential for the generation of the first mycobacterial genome sequence, *M. tuberculosis* H37Rv (Philipp *et al*, 1996; Cole *et al*, 1998). These studies also confirmed the extensive relatedness and sequence variation amongst genomes of mycobacteria (Zhang *et al*, 1995; Alcaide *et al*, 1997; Legrand *et al*, 2001). PFGE has also been used as a molecular typing tool for *M. kansasii* in a number of studies (Alcaide *et al*, 1997; Iinuma *et al*, 1997; Picardeau *et al*, 1997; Zhang *et al*, 2004).

The next major step in the development of comparative genomics involved the use of genomic subtractive hybridisation, which led to the identification of Regions of Difference (RD) amongst some mycobacterial strains (Mahairas *et al*, 1996; Gordon *et al*, 1999). Using subtractive hybridisation, Gordon and co-workers were able to identify three regions of difference (RD1-RD3) in the genome of *M. tuberculosis* H37Rv that were absent from *M. bovis* BCG (Gordon *et al*, 1999). RD1 was found to be present in all virulent laboratory and clinical strains of *M. bovis* and *M. tuberculosis* (Mahairas *et al*, 1996). This region comprises 9 genes (Rv3871 – Rv3879) and spans a 9.5kb region. In *M. bovis* BCG, RD1 completely removes 7 genes (Rv3872 – Rv3878) and truncates two others (Rv3871 – Rv3879c) (Lewis *et al*, 2003). Two of the genes in this region are

involved in the secretion of two highly antigenic proteins, ESAT-6 and CFP-10, and deletion mutants of *M. tuberculosis* have been found to be less virulent than the wild type strains (Lewis *et al*, 2003). Recently, RD1 has also been found to present in *M. kansasii* and other slow-growing mycobacteria, and may play a vital role in their virulence (Arend *et al*, 2005; van Ingen *et al*, 2009).

In recent years a number of subtractive hybridisation approaches for the identification of genetic differences between genetically-related bacteria have been developed. One of its first applications in mycobacteriology involved the detection of mRNA either from an organism grown under different growth conditions or from two strains of the same or closely related species. In a recent approach, Selective Capture of Transcribed Sequences (SCOTS), a PCR-based method that directly identifies bacterial genes specifically expressed in different environments, mRNA was isolated from an experimental condition, in this case, from infected human macrophages. After converting it to complementary DNA (cDNA), the collection of molecules was prehybridised to itself to remove highly abundant sequences, after which it was hybridised to biotinylated bacterial genomic DNA. The captured DNA molecules were then isolated via streptavidin-coated magnetic beads, eluted and subjected to a series of SCOTS to enrich for the targeted sequences (Graham and Clark-Curtiss, 1999). The approach has provided insights into several important microbial pathogens and allowed the first global characterisation of bacterial gene expression patterns in naturally infected human tissues. SCOTS has been used to identify potentially important genes in *M. tuberculosis* (Graham and Clark-Curtiss, 1999), *Salmonella typhimurium* (Morrow *et al*, 1999), *Helicobacter pylori* (Graham *et al*, 2002), *Listeria monocytogenes* (Liu *et al*, 2002) and *M. avium* (Hou *et al*, 2002). Although SCOTS has the advantage of identifying low abundance mRNA, it cannot be used to

quantify *in vivo* gene expression levels because of the cDNA normalisation step included in the protocol. Additionally, it does not detect global genetic variations amongst bacterial strains or species but targets cDNA derived from differentially expressed genes.

To facilitate the detection of genetic variations amongst phylogenetically-related bacteria, a variation of the SCOTS method that does not rely on mRNA expression, termed Genome Fragment Enrichment (GFE) was recently developed (Shanks *et al*, 2006a). Like SCOTS, GFE is a positive selection strategy that utilises competitive solution hybridisation to obtain DNA fragments present in one pool of fragments but not another. Biotinylated, sheared genomic DNA from one bacterial strain or species (tester) was prehybridised with sheared genomic DNA from a second strain or species (driver) prior to being self-hybridised with PCR amplified DNA sequences from the first bacterium containing defined terminal sequence tags. This step blocked complementary sequences between the two populations. The desired DNA hybrids consisting of original biotinylated strands not blocked by this step were then isolated by streptavidin binding and, the captured genomic fragments containing defined terminal tags were selectively amplified by PCR. GFE has been successfully applied to the identification of *Enterococcus faecalis* genome-specific sequences (Shanks *et al*, 2006a), bovine faeces-specific DNA sequences (Shanks *et al*, 2006b) and bacterial DNA markers for the detection of chicken and human faecal pollution in water (Lu *et al*, 2007; Shanks *et al*, 2007).

Another recent and simplified alternative to the identification of chromosomal variations in closely related organisms is Hybridisation-Monitored Genome Differential Analysis (HMDA). This is a PCR-based solid subtractive hybridisation method that utilises a ribosomal DNA (rDNA) monitoring system to track the entire subtraction process

(Yueqing *et al*, 2006). In the original approach, tester DNA was enzymatically digested, tagged with adaptors for PCR amplification and exposed to randomly sheared driver DNA immobilised on a nylon membrane disc to eliminate homologous fragments. Several rounds of the hybridisation process were carried out with the replacement of the driver discs after each round. The end-point of the hybridisation process was determined when the conserved 18S rDNA sequence in the tester DNA population was no longer detectable by PCR. The resulting unbound tester DNA fragments were then PCR-amplified and directly cloned for further analysis (Yueqing *et al*, 2006). HMDA has been successfully used to identify unique sequences found in *Saccharomyces cerevisiae* but absent in *Schizosaccharomyces pombe* (Yueqing *et al*, 2006).

However, there is very little information available regarding genetic studies on *M. kansasii.* Efforts are currently underway by the McGill University Sequencing Centre in Canada to determine the complete genome sequence of its type strain, *M. kansasii* ATCC 12478. Preliminary data generated from the draft genomic sequence, completed on December 16, 2008, shows that the genome of *M. kansasii* is about 6.4 Mb and contains approximately 5961 genes and 5913 proteins from 299 DNA contiguous sequences (contigs) http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome&cmd=Retrieve&dopt=Overview&list_uids=6107 [last accessed June 1, 2010]). The data being generated from this sequence will shed some light on its genetic characteristics, such as those related to virulence and its evolution (Behr, 2008).

1.6 Mode of Infection and Immune Response

Unlike *M. tuberculosis*, there is very little information available on how infection by *M. kansasii* is acquired. Infection is probably achieved through inhalation of *M. kansasii*-

containing aerosols into pulmonary alveoli or implantation through broken skin. Generally, there is interplay between the mycobacteria and the host to determine the outcome of infection. *M. kansasii*, like other mycobacteria, is an intracellular pathogen, infecting and surviving in macrophages and re-directing host responses to make a safe haven for itself (Smith *et al*, 2003). Following infection, the mycobacteria bind to phagocytic receptors and are taken up by resident macrophages, dendritic cells and monocytes recruited from the blood stream. The mycobacteria can also be trafficked to other sites, especially in immunocompromised patients, where they generally persist and multiply (McGeady and Murphey, 1981; Paniker, 2007).

1.6.1 Innate Immune Response

Endocytosis of mycobacteria involves different receptors on phagocytic cells, which bind either to non-opsonised mycobacteria or recognise opsonins on the surface of mycobacteria. *In vitro* studies have implicated several surface receptors on phagocytic cells involved in the recognition and binding of mycobacteria. These include complement receptors (CR) 1, 2, 3, and 4, mannose receptors (MR), CD14, pulmonary surfactant protein A (Sp-A) and Toll-like receptors (TLR) and scavenger receptors (Schlesinger *et al*, 1996; Means *et al*, 1999; Le Cabec *et al*, 2000; Peyron *et al*, 2000). The CR3 receptor has been shown to play a key role in the phagocytosis of *M. kansasii*, both by macrophages and neutrophils (Le Cabec *et al*, 2000; Peyron *et al*, 2000). Cholesterol seems to play an essential role in the internalisation of the mycobacteria by accumulating around phagocytic receptors prior to the uptake of the mycobacteria (Gatfield and Pieters, 2000; Peyron *et al*, 2000).

Immune cells are able to recognise the mycobacteria via molecules present on the surface of the bacteria termed pathogenic-associated molecular patterns (PAMPs), molecules expressed invariably by several related pathogens but not by normal host cells (Medzhitov and Janeway, 2002). PAMPs are ligands for innate pattern recognition receptors that are expressed on myeloid antigen presenting cells (APC), macrophages and dendritic cells. APCs utilise PAMP receptors to recognise pathogens, initiate and orchestrate inflammatory responses to trigger mechanisms of pathogen killing via innate immunity. Antigens derived from engulfed pathogens are subsequently presented to lymphocytes, which initiate adaptive immune responses. The human pattern receptors include a family of 11 Toll-like receptors (TLRs), among which TLR2 and TLR4 are cell surface proteins specialised in binding to lipid-based components of mycobacterial cell walls and initiate an inflammatory signalling cascade that is critical to the innate immune response (O'Neill and Bowie, 2007). Recognition of *M. kansasii* has been reported to engage TLR2 (Vignal *et al*, 2003; Quesniaux *et al*, 2004), while that of *M. tuberculosis* involves both TLR2 and TLR4 (Means *et al*, 1999). TLR2 binds mycobacterial lipomannans (LM), while the ligands for TLR4 remain unknown (Jo *et al*, 2007). Other molecules such as the mannose-capped lipoarabinomannans (ManLAM) do not act via TLR but bind to the MR and dendritic cell-specific intracellular adhesion molecule-grabbing non-integrin (DC-SIGN) (Jo *et al*, 2007).

The host defence mechanism against mycobacterial infection mainly employs TLR2 and TLR4 (Jo *et al*, 2007). The activation of these two receptors affects the type 1 helper (Th1) and type 2 helper (Th2) lymphocytes differently. One of the key signalling pathways activated by these receptors is through the nuclear factor-κB (NF-κB), which leads to the expression of cytokines, chemokines and inducible nitric synthase (iNOS) (Jo *et al*, 2007).

iNOS generates nitric oxide (NO), a key mediator in reactive nitrogen intermediates mediated by microbicidal mechanisms of macrophages (Fang, 2004). Activation of NF-κB by both TLR2 and TLR4 requires the adaptor protein, myeloid differentiation primary response gene 88 (MyD88), which is important for the induction of Tumour Necrosis Factor-α (TNF-α), interleukin-10 (IL-10), IL-18, interferon-γ (IFN-γ) and IL-1β (Ryffel *et al*, 2006; O'Neill and Bowie, 2007; Fang *et al*, 2010). Stimulation of TLR4 by mycobacteria has also been shown to induce production of IL-32, a cell-associated pro-inflammatory cytokine produced through a caspase-1 and IL-18 dependent production of IFN-γ and its main sources are Natural Killer (NK) cells, epithelial cells and blood monocytes (Netea *et al*, 2006). Additional studies have demonstrated that in human monocytes and macrophages TLR2 also triggers the expression of vitamin D receptor and vitamin D-1-hydroxylase genes, leading to the induction of an antimicrobial peptide, cathelicidin, which results in the killing of mycobacteria (Liu *et al*, 2007).

CCR5, a chemokine receptor found on dendritic cells, is another important host molecule that could act as a pattern recognition receptor and has been shown to recognise mycobacterial heat shock protein 70 (hsp70), leading to diverse cellular immune responses (Floto *et al*, 2006).

Apart from macrophages and dendritic cells, other leukocytes also participate in the innate immune responses, including neutrophils and NK cells. Neutrophils are amongst the earliest cells to be recruited to sites of inflammation. They have well-characterised antimicrobial mechanisms (Urban *et al*, 2006). Using whole blood assays, neutrophils were shown to contribute significantly to innate resistance to mycobacterial infection and this resistance was attributed to the presence of antimicrobial peptides in these cells

(Martineau *et al*, 2007). Conversely, neutrophils have also been associated with the development of disease rather than protection of the host. Eruslanov and co-workers, using a mouse model, demonstrated that *M. tuberculosis*-susceptible mice had larger and longer accumulation of neutrophils in tuberculous lesions as compared to *M. tuberculosis*-resistant mice (Eruslanov *et al*, 2005). This may have been influenced by the differential expression of chemoattractant molecules to neutrophils (Keller *et al*, 2006).

NK cells are also very important effector cells of the innate immune system (Vankayalapati and Barnes, 2009). They act by directly lysing pathogens or infected cells by the production of perforins and are amongst the first cells to produce IFN-γ during immune response. In TB, NK cells have also been shown to contribute to the clearance of *M. tuberculosis* through production of IL-22, which inhibits intracellular mycobacterial growth by enhancing phagolysosomal fusion (Dhiman *et al*, 2009) and to optimise the ability of CD8+ cells to produce IFN-γ and lyse *M. tuberculosis*-infected cells (Vankayalapati and Barnes, 2009).

1.6.2 Adaptive Immune Response

Unlike the innate immune mechanisms, the adaptive immune responses require specific recognition of foreign antigens. After the innate system recognises an invading pathogen and attempts to control its early spread, the adaptive immune response is activated to deal with the pathogen in a specific manner and is effected through the humoral and cell-mediated immune responses.

1.6.2.1 Humoral Immune Response

Since mycobacteria are intracellular pathogens, antibodies may not gain access to the pathogens and may not play a protective role. However, during the initial stages of infection, antibodies seem to have an opsonising role by improving phagocytosis by macrophages or the cytotoxic actions of NK cells. In a recent study De Vallière and co-workers demonstrated that lipoarabimannan (LAM)-specific antibodies increased the internalisation and killing of *M. bovis* BCG. Furthermore, the *M. bovis* BCG-induced antibodies were shown to significantly enhance the cell-mediated immune response with increased T cell proliferation and IFN-γ production in mycobacterium-specific $CD4^+$ T lymphocytes ($CD4^+$ T cells) and CD8+ T cells (de Valliere *et al*, 2005). This is a good example through which mycobacterium-specific antibodies have been shown to have the potential of enhancing both innate and cell-mediated immune responses to mycobacteria.

Recently, the use of passive immunisation with immunoglobulin A (IgA) antibodies and IFN-γ was attempted in order to induce protection against TB using an experimental mouse model of TB lung infection. IgA and IFN-γ were administered three days prior to infection and a further co-inoculation with IgA after 2 hrs, 2 days and 7 days after an aerosol infection with *M. tuberculosis* H37Rv. A 17-fold reduction in colony-forming units (CFU), as wells as a lowering of granulomatous infiltration of the lungs, were observed (Reljic *et al*, 2006).

1.6.2.2 Cell-Mediated Immune Response

Control of mycobacterial infection by the host depends on a functional cell-mediated immune response through the $CD4^+$ T cells. In essence, the initiation of the adaptive immune responses is influenced by antigen presentation, co-stimulation signals from

mycobacteria and cytokine production. Since mycobacteria reside inside a compartment within macrophages, their antigens are presented in association with Major Histocompatibility Complex (MHC) class II molecules to specific $CD4^+$ T cells (Reith and Mach, 2001). MHC class I molecules, expressed on all nucleated cells, present mycobacterial antigens that escape from phagosomes to $CD8^+$ T cells and non-polymorphic MHC class I molecules such as type CD1 molecules, expressed on macrophages and dendritic cells, are capable of presenting mycobacterial lipoproteins to CD1-restricted T cells (Munk and Emoto, 1995). The expression of these antigen presenting molecules is regulated by cytokines. IFN-γ stimulates the expression of MHC (Reith and Mach, 2001) while anti-inflammatory cytokines inhibit its expression. Macrophages infected with mycobacteria are capable of down-regulating the expression of MHC molecules through production of anti-inflammatory cytokines (Weiss *et al*, 2001).

Macrophages play a role in the initial defence against mycobacteria by producing cytokines that have a regulatory role on macrophages themselves and T cells, which are important in specific cell-mediated immune responses (Flynn and Chan, 2001). They produce IL-12, IL-10 and IL-18, which strongly influence the T cell cytokine responses (Oppmann *et al*, 2000; Luo *et al*, 2004). IL-12 is a potent stimulus for T cells to secrete IFNγ, whereas IL-10 inhibits the production of IL-12 and favours the development of cells that produce T helper 2 (Th2) cytokines. IL-18 acts synergistically with IL-12 to enhance T cell production of IFNγ (Luo *et al*, 2004).

$CD4^+$ T cells contribute to defence against infectious agents by producing specific patterns of cytokines. Based on their functions, pattern of cytokine secretion and their expression of specific transcription factors, four $CD4^+$ T cell effector lineages have been described:

Th1, Th2, Th17 and T regulatory (T reg) cells (Murphy and Reiner, 2002; Sakaguchi, 2004; Harrington *et al*, 2005; Bettelli *et al*, 2007; Bluestone *et al*, 2009). Other Th lineages may exist (Szabo *et al*, 2003; Bluestone *et al*, 2009). Th1 cells mainly produce IFNγ (important for macrophage activation and clearance of intracellular pathogens) and IL-2, whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13, which are important for the production of IgE, eosinophil recruitment and clearance of extracellular parasites (Szabo *et al*, 2003; Ansel *et al*, 2006).

Although it is well recognised that Th1 cytokines are critical in cell-mediated responses to mycobacteria, it is also clear that this immunity alone is not enough (Flynn, 2004). Recent data seem to indicate that Th1 cells and IFN-γ play a limited role in murine lung infection with *M. kansasii* (Wieland *et al*, 2006a). Both wild-type mice and knock-out mice were shown to display normal resistance against pulmonary infection with *M. kansasii*. This was in sharp contrast to previous findings that showed that Th1 cells were important for protection against *M. tuberculosis* (Flynn and Chan, 2001). In a follow-up study, IL-1 was shown to positively contribute to an effective clearance of *M. kansasii* from the respiratory tract by an unidentified mechanism (Wieland *et al*, 2006b). It is thought that TLRs, complement and antibodies could be involved in the activation of IL-1 and that TNF-α could have augmented this protective mechanism since it is one of the key mediators of host defence mechanisms against mycobacterial infection (Flynn and Chan, 2001). TNF-α is important for walling off infection and prevention of dissemination (Flynn and Chan, 2001).

Th17 cells belong to a $CD4^+$ T cell subset that is distinct from Th1 and Th2 subsets. They have significant pro-inflammatory functions via the production of cytokines IL-17A, IL-

17F, IL-21 and IL-22 (Harrington *et al*, 2005; Park *et al*, 2005; Liang *et al*, 2006). Through the production of these pro-inflammatory cytokines, Th17 cells also trigger the production of other cytokines such as IL-6, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Granulocyte Colony-Stimulating Factor (G-CSF), chemokines (CXCL-1, CXCL-2, CXCL-5, CXCL-8) and metalloproteins (Liang *et al*, 2006; Chen *et al*, 2010). Th17 cells play a significant role not only in the development of autoimmune and inflammatory diseases, but also in protection against extracellular bacteria and fungi, particularly at epithelial surfaces (Weaver *et al*, 2007). Recent studies have also indicated that Th17 cells are the major IL-17-producing cells and participate in the protective immunity against *M. tuberculosis* (Wozniak *et al*, 2006; Khader *et al*, 2007). Khader and colleagues reported an indirect role for the Th17 response in protective immunity in mouse models of *M. tuberculosis* infection (Khader *et al*, 2005, Khader *et al*, 2007; Khader and Cooper, 2008). Human Th17 response is inducible by *M. tuberculosis* infection and a reduced *M. tuberculosis* antigen-specific Th17 response, which may be due to suppression of Th1 cytokine, has been observed in patients with active disease compared to healthy subjects (Scriba *et al*, 2008).

Regulatory T cells (T regs) are characterised by expression of the transcription factor forkhead box P3 (FOXP3) and IL-2 and Transforming Growth Factor-β (TGF-β), and CD28 co-stimulation seem to be important for their generation (Chen *et al*, 2003). These cells may be involved in self-tolerance, immune regulation and promotion of immune responses under certain circumstances (Curotto de Lafaille and Lafaille, 2009).

The destruction of intracellular pathogens primarily relies on cytotoxic lymphocytes such as NK cells and CD8+ T cells. CD8+ T cells require an antigen to be presented in

association with MHC class I molecules but it is not clear how the activation of these cells occurs as bacterial antigen presentation is not likely to occur via MHC class I proteins. One hypothesis states that mycobacterial antigens either leak out from, or are actively transported out of, the phagosome into the cytosol where they are processed for MHC class I presentation (Hartmann and Plum, 1999). A second hypothesis assumes that the presentation of mycobacterial antigens is done in the context of MHC molecule Ib, which does not require processing of antigens through the endoplasmic reticulum (Munk and Emoto, 1995). Whatever the mechanism may be, cytotoxic CD8+ T cells have been shown to secrete IFN-γ and TNF-α, and are capable of directly killing mycobacteria-containing macrophages through the production of perforins and granzymes that punch holes in the cell membranes (Stenger *et al*, 1997). Additionally, they may also produce granulysin which enter infected macrophages through holes and directly kill intracellular mycobacteria (Serbina and Flynn, 1999).

In additional to T cells discussed above, large granular lymphocytes, referred to as γδ T cells, play a key role in adaptive immune response. These cells do not require interaction with MHC molecules for activation and function as cytotoxic cells, and may act as antigen presenting cells (Kabelitz *et al*, 2000; Brandes *et al*, 2005). Stimulation of human γδ T cells has been shown to result in aggressive production of IFN-γ and TNF-α, which are potent activators of phagosome-lysosome fusion (Boom, 1999). In both human and mouse, γδ T cells have been shown to contribute to the elimination of *M. tuberculosis* and to have an anti-inflammatory effect (D'Souza *et al*, 1997; De La Barrera *et al*, 2003). However, the role of these cells in *M. kansasii* infection is unknown.

1.6.2.3 Granulomas

One of the major outcomes of mycobacterial infection *in vivo* is the formation of granulomas. Granulomas are sites for containment of mycobacteria consisting of a central necrotic core surrounded by concentric layers of macrophages, Langerhans giant cells and lymphocytes (Flynn and Chan, 2001). Mature granulomas are characterised by high levels of immunological activity mediated by macrophages and T cells resulting in the production of a variety of cytokines and chemokines (Flynn and Chan, 2001). However, mycobacteria have evolved strategies to evade complete eradication in the granulomas. When the immune response is weakened due to immunosuppression, the bacilli within the granulomas can escape from the containment area to cause active disease and dissemination to other sites (Smith *et al*, 2003). Only limited work has been done to understand immune responses elicited by *M. kansasii*.

1.7 Pathogenesis

Despite the clinical importance of *M. kansasii*, little is known about its pathogenicity. Most of the literature to date has mainly dealt with clinical aspects of *M. kansasii*-induced disease. Once inside the host cell, pathogenic mycobacteria are capable of evading destruction by the immune system. An important key to the success of these organisms is likely to be due to their unusual cell wall structure, comprising a highly complex array of lipids, glycolipids and proteins, and its interaction with the immune system. Two of the predominant glycolipids, lipoarabinomannans (LAM) and their biosynthetic precursors, lipomannans (LM) are believed to play important roles in the physiology of the bacterium as well as in the modulation of the host response during infection. LAMs have been reported to be secreted from macrophages and, therefore, may also affect the functioning of uninfected neighbouring cells in a paracrine manner (Beatty *et al*, 2000). Using the

THP-1 human monocytic cell line, it was shown that *M. kansasii* LM (kanLM) was a potent inducer of apoptosis and pro-inflammatory cytokines, highlighting the importance of kanLM in the pathogenesis of *M. kansasii*. Assessment of the role of *M. kansasii* LAM (kanLAM) showed that it did not induce apoptosis, but only did so after the removal of the arabimannan domain (Guerardel *et al*, 2003; Vignal *et al*, 2003). The presence of oligomannoside caps on LAM is considered to be one of the strategies that pathogenic mycobacteria like *M. kansasii* and *M. tuberculosis* utilise to mimic endogenous host components in order to evade host defence mechanisms (Jozefowski *et al*, 2008). In fact, it has been shown that the presence of ManLAM on *M. tuberculosis* stimulates antigen presenting cells (APC) to induce regulatory T cells to start producing IL-10 and TGF-β, two of the cytokines that significantly contribute to the inhibition of Th1 responses (Garg *et al*, 2008).

In immunocompetent patients, pulmonary disease has been reported to generally resemble that of TB and this includes formation of cavitary lesions, which mostly involve upper lobes (Shitrit *et al*, 2007). Using BALB/c mice, Fujita and co-workers were able to demonstrate that trehalose 6,6'-dimycolate (TDM), the cord factor, from both *M. kansasii* and *M. tuberculosis* induced marked toxicity, thymic atrophy and granulomatogenicity in lungs and spleens of the mice as compared to TDMs from other mycobacterial strains. This was mainly attributed to the existence of longer chain and abundance of α-mycolic acids in TDMs of these two mycobacterial species (Fujita *et al*, 2007). In some instances *M. kansasii* infection has been shown to mimic other disease conditions. Chen and co-workers, in a recent case report, showed that two immunocompetent patients with systemic *M. kansasii* infection presented with disease resembling a peripheral T cell lymphoma. These patients presented with nodules infiltrated with lymphocytes and small

aggregates of epithelioid histiocytes. In both cases, granulomas were poorly formed, mimicking peripheral T cell lymphomas, but were negative for T cell receptor (TCR) gene rearrangement (Chen *et al*, 2008). Without the isolation and identification of *M. kansasii*, diagnosis of this *M. kansasii*-induced disease would have been complicated.

In patients with HIV infection, *M. kansasii* disease presents with diverse features which may make diagnosis difficult (Smith *et al*, 2003; Cattamanchi *et al*, 2008). A recent review of pathological features associated with *M. kansasii* infection in patients with AIDS showed that *M. kansasii* infection induced predominantly pulmonary disease with a wide range of inflammatory reactions. This included granulomas with or without necrosis, neutrophilic abscesses, spindle cell proliferations and foci of granular eosinophilic necrosis. A high incidence of thoracic lymph node involvement and a lower incidence of disseminated disease were also reported (Smith *et al*, 2003). This is probably the only study that documents detailed pathological features associated with *M. kansasii*-induced disease.

1.8 Clinical Presentations

M. kansasii infections are more common in males than in females, especially in the older population, and age shifts correlate with the population infected with HIV (Churchyard, 2000; Churchyard and Grant, 2000; Yim *et al*, 2005). The high frequency of infection observed in males may be attributed to their occupation, which may predispose them to high risk factors for *M. kansasii* infection (Wolinsky, 1992; Bloch *et al*, 1998; Corbett *et al*, 1999; Jacobson *et al*, 2000; Churchyard and Corbett, 2001).

1.8.1 Disease in Immunocompetent Patients

In immunocompetent patients, pulmonary disease is the most frequent clinical manifestation but in some patients there is no associated illness (Wolinsky, 1992; Bloch *et al*, 1998; Griffith, 2002; Arend *et al*, 2004; Yim *et al*, 2005; Shitrit *et al*, 2006). The antigenic and clinical characteristics of *M. kansasii* make it the mycobacterium most similar to *M. tuberculosis*, but the symptoms it causes may be less severe and more chronic as compared to that induced by *M. tuberculosis* (Bloch *et al*, 1998; Koh *et al*, 2002; Marras and Daley, 2004; Shitrit *et al*, 2008). In the years preceding the emergence of the HIV/AIDS pandemic, *M. kansasii* was the NTM agent mostly responsible for pulmonary infections in patients with previous pulmonary disease, but disseminated disease was extremely rare (Horsburgh, 1996).

Lung disease affects more than 50% of patients and, if untreated, can lead to death (Griffith, 2002; Shitrit *et al*, 2007). Symptoms closely resemble those of pulmonary tuberculosis. Commonly reported symptoms include fever, chills, night sweats, productive or non-productive cough, weight loss, fatigue, dyspnoea and chest pain (Bloch *et al*, 1998; Koh *et al*, 2002). Chest radiography abnormalities in the majority of patients show cavitary disease mainly in the right lung with an upper lobe predilection, usually with evidence of cavitation and scarring (Griffith, 2002; Shitrit *et al*, 2008). Extrapulmonary infections, including lymphadenitis, skin and soft tissue infection and joint infection, have been reported occasionally (Horsburgh, 1996; Bloch *et al*, 1998; Razavi and Cleveland, 2000; Koh *et al*, 2002; Benton *et al*, 2004). Disseminated infections are rare in immunocompetent patients (Wolinsky, 1992; Griffith, 2002).

1.8.2 Disease in HIV-infected patients

M. kansasii infection usually manifests late in the course of HIV disease (Klein *et al*, 1998; Manfredi *et al*, 2004). In patients infected with HIV, any organ can be affected, making the clinical presentation non-specific. Commonly reported symptoms include fever, night sweats, weight loss, fatigue, cough and chest pain (Witzig *et al*, 1995; Cattamanchi *et al*, 2008). Chest radiographs mainly show lack of cavitations. The most common features are consolidations and nodules in the mid and lower lung zones (Cattamanchi *et al*, 2008). In the same study of radiographical presentations of *M. kansasii* lung disease in patients with HIV, Cattamanchi and co-workers found that patients with HIV infection and *M. kansasii* lung disease present with diverse chest radiological patterns, most commonly consolidations and nodules mostly located in the mid and lower lung zones. This is in contrast to the findings in patients without HIV in whom upper-lobe radiographic abnormalities are normally seen (Griffith, 2002; Shitrit *et al*, 2007).

In predicting the mortality of pulmonary *M. kansasii* infection in patients with HIV, Marras and colleagues studied 127 patients with pulmonary *M. kansasii* and found that 33% fulfilled the American Thoracic Society criteria for the disease (Griffith *et al*, 2007). Twenty-one percent of these received at least one antimycobacterial drug for at least 3 months and 53% died. Mortality was associated with a lower CD4 cell count and positive smear microscopy. Antiretroviral therapy and *M. kansasii* treatment were associated with survival (Marras *et al*, 2004).

1.9 Diagnosis

It is generally accepted that the isolation of NTMs from sputum of individual patients is not proof of disease, as this may represent colonisation of the respiratory tract or specimen

contamination. This has led to the establishment of the diagnostic criteria for NTM by the American Thoracic Society (ATS) (Griffith *et al*, 2007). These globally adopted criteria have also been applied to *M. kansasii* and are based the isolation of NTM from two or more sputum specimens taken at different times in addition to clinical and chest radiographical abnormalities.

However, these criteria are not considered appropriate for the diagnosis of NTM disease in the South African mining industry as they have demonstrated under-diagnosis of *M. kansasii* disease due to the logistical barriers associated with the requirement for multiple sputum isolates (Churchyard and Corbett, 2001). To address this problem, the Safety In Mines Research Advisory Committee (SIMRAC) in South Africa has suggested a number of modification to the ATS diagnostic criteria for pulmonary NTM disease in the South African mining industry (Churchyard and Corbett, 2001). The following are the suggested adaptations of the ATS diagnostic criteria:

- compatible signs and symptoms and reasonable exclusion of other disease to explain the condition (including *M. tuberculosis* disease)
- pulmonary changes compatible with pulmonary NTM disease
- two NTM isolates from separate sputum or broncho-alveolar lavage (BAL) specimens. The ATS recommends at least 3 isolates to confirm smear negative disease
- heavy growth and numerous acid-fast bacilli (AFB) isolated for a single available BAL specimen
- single NTM isolate from lung biopsy tissue; single available sputum isolate identified as *M. kansasii*. The ATS recommendations do not distinguish between species with different intrinsic pathogenesis and do not recommend treating a

single sputum or BAL NTM isolate unless the diagnosis is supported by granulomas or AFB on lung biopsy tissue

- single available sputum isolate identified as an NTM species associated with a previous episode of mycobacterial disease (potential relapse).

Generally, diagnosis of NTM disease, including that of *M. kansasii* is based on clinical, radiological and laboratory findings. The confirmation of clinical and radiological suspicions of *M. kansasii*-associated disease involves the use of both phenotypic and genotypic methods. Phenotypic methods are used to detect visible characteristics of the organism such as acid-fastness, colonial morphology and biochemical reactions. By contrast, genotypic methods detect the genetic characteristics of the organism.

1.9.1 Phenotypic Methods

On culture *M. kansasii* exhibits slow growth and photochromogenicity by producing a yellow pigment upon exposure to light, grows optimally at 37^{o}C and produces either rough or smooth colonies with irregular edges on solid media (Wayne and Kubica, 1986). Its isolation involves the use of media such as Löwenstein-Jensen (LJ), Middlebrook 7H10 (M7H10) and M7H9 broth. Optimised automated techniques such as the BACTEC MGIT 960 (Becton Dickinson Laboratories, Sparks, MD, USA) are also used and these systems have reduced the time taken for detecting mycobacteria. The main biochemical tests used for its identification include production of catalase, urease, pyrazinamidase, nitrate reductase production and its ability to hydrolyse Tween 80 (Kent and Kubica, 1985; Leão *et al*, 2004). Chromatographic methods such as High Performance Liquid Chromatography (HPLC) have also been shown to be useful for the definitive

identification of *M. kansasii* (Riviere *et al*, 1987; Butler and Kilburn, 1988; Butler and Guthertz, 2001).

1.9.2 Genotypic Methods

A number of molecular techniques have been developed for the detection and identification of mycobacteria and are now providing rapid and accurate results. These techniques are mainly based on the amplification or hybridisation of nucleic acids.

1.9.2.1 PCR Restriction-Enzyme Analysis

PCR Restriction-Enzyme Analysis (PRA) is a rapid PCR method, based on the amplification of a 441bp DNA fragment of the *hsp*65 gene, followed by digestion with two restriction enzymes, *BstE*II and *Hae*III (Telenti *et al*, 1993). The products of the digestion are analysed by agarose gel electrophoresis, evaluated with the help of a published algorithm (Devallois *et al*, 1997; Chimara *et al*, 2008) and compared to PRASITE (http://app.chuv.ch/parasite/index.html), an internet database maintained by the Swiss National Centre for Mycobacteria. PRA analysis of *M. kansasii* results in seven subtypes. Other genes, such as *rpoB* and *gyrB*, have also been used with PRA for the identification of mycobacteria (Lee *et al*, 2000; Goh *et al*, 2006). A number of studies have reported the successful application of this assay in clinical settings (de Magalhaes *et al*, 2002; Leão *et al*, 2005, Martin *et al*, 2007).

1.9.2.2 Commercial Assays

A number of molecular assays for identifying *M. kansasii* and other mycobacteria are available commercially and are widely acknowledged in literature. These include AccuProbe (Gen-Probe, San Diego, CA, USA [Drake *et al*, 1987; Reisner *et al*, 1994;

Tortoli *et al*, 1996; Richter *et al*, 1999]), Inno LiPA Mycobacteria (Innogenetic, Ghent, Belgium [Scarparo *et al*, 2001; Mijs *et al*, 2002; Tortoli *et al*, 2003b]) and GenoType Mycobacterium (Hain Life Science, Nehren, Germany [Richter *et al*, 2006; Franco-Alvarez de Luna *et al*, 2006; Gitti *et al*, 2006; Russo *et al*, 2006; Neonakis *et al*, 2007; Seagar *et al*, 2008]).

1.9.2.3 DNA Sequencing

DNA Sequencing is regarded as the "gold" standard for the identification of all microorganisms, including mycobacteria. The rationale behind this methodology is that highly conserved target regions, but variable genes within the *Mycobacterium* genus, are first amplified by PCR, followed by sequencing of the amplicons on an automated sequencer. Once the sequences have been determined, they are compared with a library of known sequences in databases available on the internet. Genes commonly targeted for sequencing include the *16S rDNA* gene (Rogall *et al*, 1990a; Stahl and Urbance, 1990; Kox *et al*, 1995; Harmsen *et al*, 2002) the *16S-23S rDNA* internal transcribed spacer (ITS) (Roth *et al*, 2000; Iwamoto and Saito, 2006), the *rpoB* gene (Gingeras *et al*, 1998; Kim *et al*, 1999), the *gyrB* gene (Kasai *et al*, 2000), the *hsp65* gene (Telenti *et al*, 1993; Ringuet *et al*, 1999; Brunello *et al*, 2001), the *recA* gene (Blackwood *et al*, 2000), the *gyrA* gene (Sander *et al*, 1998), the *sodA* gene (Zolg and Philippi-Schulz, 1994) and the *secA1* gene (Zelazny *et al*, 2005). This methodology, generally, offers microbiology laboratories the ability to identify newly described mycobacterial species.

1.10 Treatment

M. kansasii isolated from lungs or elsewhere almost always warrants treatment, especially in patients with AIDS and in other immunocompromised groups, as it is more likely to be

associated with disease (Bloch *et al*, 1998, Corbett *et al*, 1999a; Churchyard and Corbett, 2001; Griffith, 2002; Marras and Daley, 2004; Cattamanchi *et al*, 2008). Unfortunately, there is no standardised treatment for *M. kansasii*, although it seems to be sensitive to a number of anti-tuberculosis medications (Griffith, 2002). The current ATS recommendation for treatment of pulmonary *M. kansasii* disease in non-HIV infected patients includes rifampicin, isoniazid and ethambutol administered daily for 18 months plus 12 months of negative sputum cultures (Griffith *et al*, 2007). This is longer than the usual treatment for tuberculosis, which usually takes about 6 to 9 months. Clarithromycin has been suggested as a reasonable alternative in patients who cannot tolerate one of these three drugs (Griffith *et al*, 2003). Other agents with useful activity against *M. kansasii* include sulphamethoxazole, amikacin, streptomycin, fluoroquinolones and rifabutin. It is recommended that patients with rifampicin-resistant *M. kansasii* strains should be treated with clarithromycin, or azithromycin, moxifloxaxin, ethambutol, sulphamethoxazole, or streptomycin (Griffith, 2007).

The recommended treatment regimen for disseminated *M. kansasii* disease is the same as for pulmonary disease but rifampicin is contra-indicated in patients with AIDS taking protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors NNRT) (Graybill and Bocanegra, 2001; Griffith *et al*, 2007). Rifampicin is a potent inducer of hepatic P-450 cytochrome enzymes, resulting in significantly enhanced metabolism of these two classes of drugs, rendering them potentially ineffective (Graybill and Bocanegra, 2001). In place of rifampicin, rifabutin has been recommended (Griffith *et al*, 2007).

1.11 Statement of the Problem and Study Justification

The incidence of *M. kansasii* disease in the South African gold-mining workforce appears to be very high, and *M. kansasii* has been reported to the most common cause of NTM lung disease in both HIV-positive and non-HIV-negative patients in this population (Churchyard *et al*, 1999; Corbett *et al*, 1999b; Churchyard, 2000; Corbett *et al*, 2000). Despite the clinical importance of *M. kansasii*, there has been no report from South Africa on the identification of its sources of infection or on any molecular or immunological analysis that would contribute to the understanding of its pathogenicity.

Such studies would contribute to the development of intervention strategies to be used in the prevention of transmission of *M. kansasii*-associated or other NTM diseases. They would also lead to the identification of genes that would be potential targets for drugs, and assist future vaccine studies.

1.12 Objectives of the Study

The primary goal of this study was to determine the molecular and immunological characteristics of clinical and environmental isolates of *M. kansasii* from a South African gold-mining region. More specifically, the objectives of the study were:

1. To identify the potential environmental sources of *M. kansasii* infection in the Vaal River gold-mining region.
2. To determine the genetic diversity amongst clinical and environmental isolates of *M. kansasii.*
3. To identify genetic differences between the predominant clinical isolate of *M. kansasii* and other environmental isolates of *M. kansasii*.
4. To determine whether *M. kansasii* has the ability to induce immunomodulation on human lymphocyte activity *in vitro*.

1.13 Thesis Layout

This thesis is divided into 7 chapters: Chapter 1 deals with the introduction and background to the Study; Chapter 2 deals with the methods used in the Study; Chapter 3 deals with aspects concerning the examination of environmental samples collected from two mining regions for the presence of mycobacteria; Chapter 4 focuses on the assessment of the genetic diversity amongst the clinical and environmental isolates of *M. kansasii*; Chapter 5 deals with the investigation of genetic differences between the clinical and environmental isolates of *M. kansasii*; Chapter 6 involves the immunological assessment of the ability of *M. kansasii* to induce immunomodulation on human lymphocyte activity *in vitro*; and Chapter 7 discusses and summarises all the major findings for the Study and suggestions for future work. The thesis layout is schematically shown in the flow chart below (Figure 1.1).

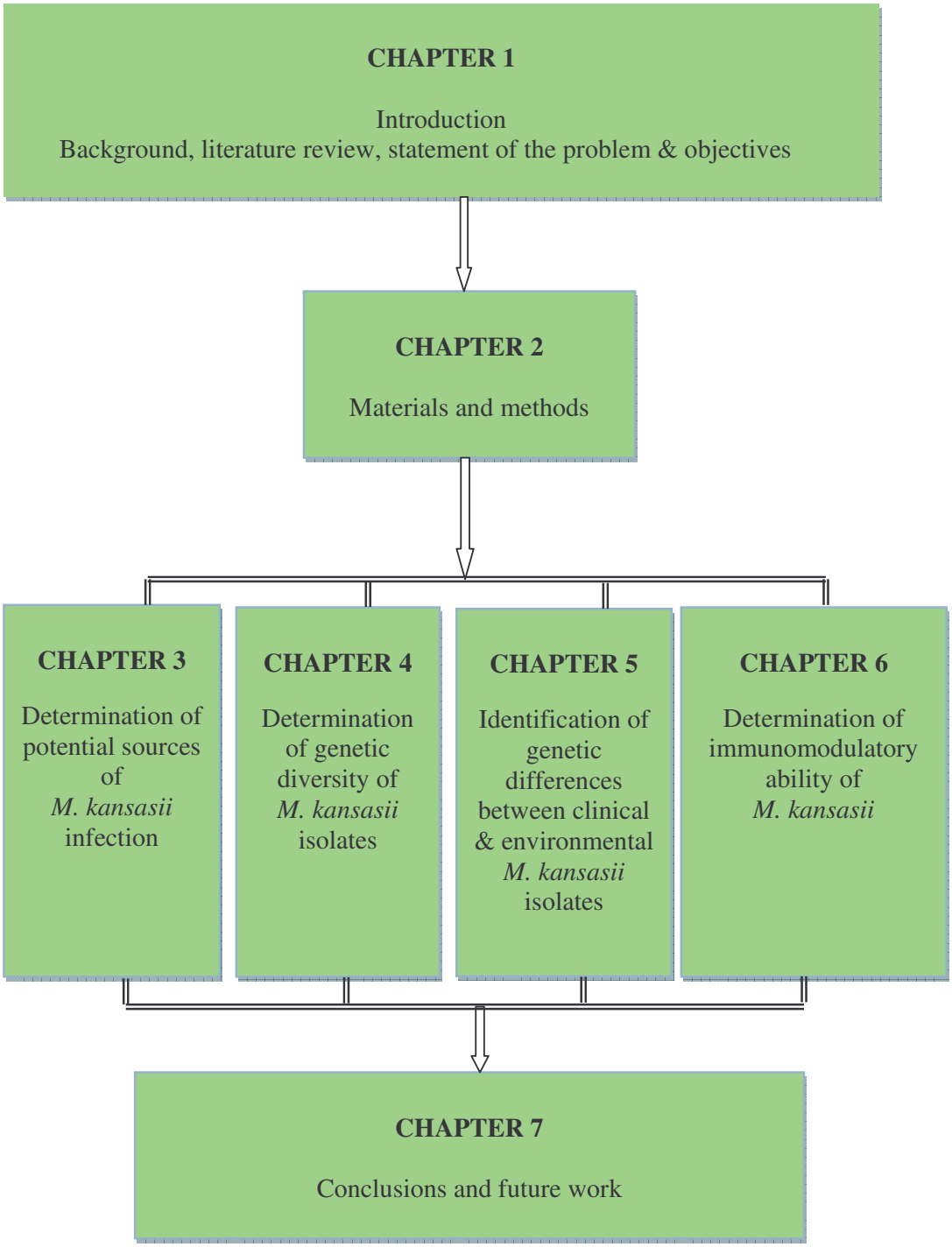


Figure 1.1: Thesis layout.

CHAPTER 2

Materials and Methods

CHAPTER 2

Materials and Methods

2.0 Introduction

This chapter is divided into four main sections that describe all the methods used in this study. The first two sections describe the bacteria, culture and DNA preparation methods used. The remaining sections describe methods used for specific chapters.

2.1 Study Sites

Water and biofilm samples were collected from a number of sites in the Vaal River gold-mining region of the North-West Province, South Africa from 31st July to 1st August 2006 and from 28th February to 2nd March 2007, and from the Secunda coal-mining complex in Mpumalanga Province from 16th April to 18th April 18 2008. Clinical isolates of *M. kansasii* were obtained through the West Vaal Hospital Tuberculosis Laboratory at Orkney situated in the Vaal River gold-mining region at the same time as the environmental samples. These isolates were obtained from the West Vaal Hospital or Leslie Williams Hospital in Cartonville. These two hospitals are the major sources of tertiary care in these regions for gold miners, who are mostly black African migrants, employed by one of the largest mining companies in South Africa. The Vaal River gold-mining region is situated at a latitude of approximately 26^{o}30'S and longitude 26^{o}45'E, some 175 km south-west of Johannesburg (Figure 2.1). The region typically experiences warm to hot summers, 22 to 34^{o}C, and cool to cold winter temperatures, with the average being about 15.5^{o}C. The topography of the region is generally flat, with some undulating hills. The Vaal River passes through this region (Figure 2.1). The second region, the Secunda coal-mining complex, is situated at a latitude of approximately 26^{o}33'S and longitude 29^{o}10'E and is

about 154 km north-east of Johannesburg. The region is situated at approximately latitude 26°33'S and longitude 29°10'E and is about 154 km north-east of Johannesburg. The region has summer temperatures ranging from 19 to 33°C and winter temperatures ranging from 6 to 26°C.

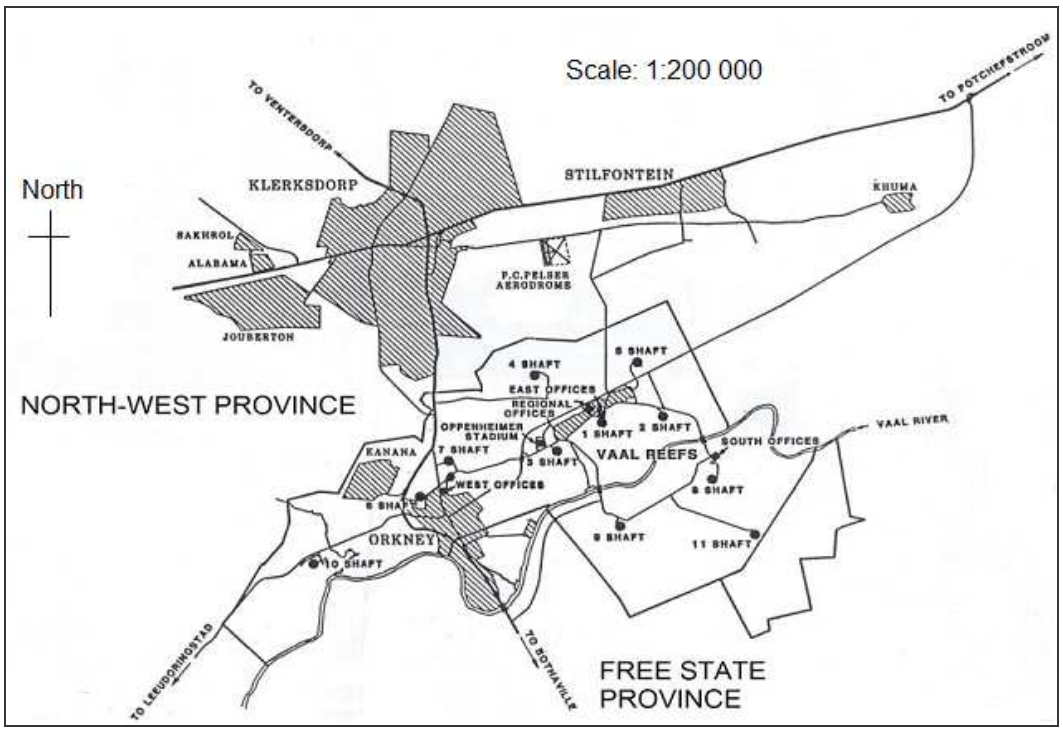


Figure 2.1: The Vaal River gold-mining region, North-West Province, South Africa, where *M. kansasii* clinical isolates, some of the water and biofilm samples were obtained. Water and biofilm samples were collected randomly from water distributing systems in mine hostels, change rooms, kitchens, boreholes, showerheads and piped underground water around a number of mine shafts shown above (Map courtesy of the Orkney Municipal Council, North-West Province).

The Vaal River gold-mining region was the main region of interest as this was the environment where a high incidence of *M. kansasii* infection had previously been reported in the gold-mining workforce (Corbett *et al*. 1999; Churchyard & Corbett 2001). The Secunda coal-mining complex was included in order to assess whether *M. kansasii* was also prevalent in a coal-mining environment. Reports from Europe have reported high incidences of *M. kansasii* infections from coal-mining regions (Kubin *et al*. 1980; Lamden *et al*. 1996; Chobot *et al*. 1997).

2.2 Bacterial Strains and Culture Conditions

Bacterial strains used in this study included clinical and environmental isolates of *M. kansasii* and *Escherichia coli* DH5α. Control strains of *M. kansasii* were kindly provided by Veroniqué Vincent and Cristina Gutierrez of the National Reference Centre for Mycobacteria, Pasteur Institute, Paris, France and Petra de Haas of the National Institute of Public Health and the Environment, Bilthoven, The Netherlands. The control strains included three isolates each for genotypes I, II, III, IV, V, one for genotype VI and ATCC 12478, obtained from the National Health Laboratory Service (NHLS) Central TB Laboratory in Johannesburg. *E. coli* DH5α (F⁻Ø80dl*ac*ZΔM15Δ[*lac*ZYA-*arg*F]U169 *RecA*1*end*A1*hsd*R17[r_k,m_k+]phoA*sup*E44*thi*-1*gyr*A96*rec*A1*ton*A (Hanahan, 1983), Invitrogen Life Technologies, Paisley, UK) was used in cloning experiments (Chapter 5).

Clinical isolates were all single patient isolates collected retrospectively and were obtained in the course of routine patient care. They were isolated from sputum samples after digestion and decontamination with the sodium hydroxide N-acetyl-L-cystein (NaOH-NALC) method (Leão *et al*, 2004). All mycobacterial strains were grown in Middlebrook 7H9 (M7H9) broth supplemented with OADC (oleic acid albumin dextrose catalase) (Difco-BD, Sparks, MD, USA), 0.5% glycerol and 0.05% Tween 80 with agitation, or on M7H10 plates supplemented with 10% OADC (Merck Chemicals, Darmstadt, Germany), 0.5% glycerol and 0.05% Tween 80, or on Löwenstein-Jensen (LJ) slopes (Diagnostic Media Products, Johannesburg). Mycobacterial strains were stored at -80^{o}C in Dubos medium (BD-Difco Laboratories, Sparks, MD, USA) (800µl Dubos stock [1.3g Dubos broth base, 30ml glycerol, 170ml distilled water] plus 800µl culture medium) until they were required for testing. *E. coli* DH5α strains were grown in Luria-Bertani (LB) broth with agitation or on LB agar plates (Sigma-Aldrich, Steinheim, Germany) supplemented

with 100µg/ml of ampicillin (Sigma-Aldrich, Steinheim, Germany). The bacteria were grown at 25, 30 or 37°C.

2.3 DNA Preparation

Genomic DNA used in this study was primarily prepared either by the thermolysis of bacteria cells in TE buffer (as described under Section 2.4.3.1) or by the CTAB method. The main method utilised for genomic DNA preparation was the modified CTAB (cetyltrimethylammonium bromide) method (Ausubel *et al*, 2001). Briefly, a loopful of mycobacterial cells from the LJ slopes were transferred into a microcentrifuge tube containing 400µl of 1x TE buffer (10mM Tris-HCl [pH 8.0], 0.mM EDTA [pH 8.0]). The cells were first heat-killed at 80°C for 20min and cooled at room temperature. Lysozyme (Sigma-Aldrich, Steinheim, Germany) was added at the final concentration of 2mg/ml and the tube was incubated overnight at 37°C. Seventy microlitres of sodium dodecyl sulphate (SDS) and 5µl of 10mg/ml proteinase K (Sigma-Aldrich, Steinheim, Germany) were added, and the mixture was incubated for 10min at 65°C. One hundred microlitres of 5M sodium chloride and 100µl CTAB/sodium chloride (4.1g sodium chloride, 10g CTAB up to 100ml distilled water) were added and incubated for 10min at 65°C. Seven hundred and fifty microlitres of chloroform/isoamyl alcohol (24:1) (Merck Chemicals, Darmstadt, Germany) were then added, vortexed and centrifuged for 5min at 14 000rpm. The aqueous supernatant was transferred to a fresh tube and 0.6 volume of isopropanol were added to precipitate the DNA. After 30min at -20°C and centrifuging for 15min at 14 000rpm, the pellet was washed once for 5min with 1ml of cold 70% ethanol, air-dried and dissolved in 50µl of 1x TE buffer. The concentration of DNA was estimated by ultraviolet spectroscopy at 260nm. A DNA sample with an optical density (OD) of 1 at 260nm corresponded to a DNA concentration of 50µg/ml of double-stranded DNA. The purity of

the DNA was determined by a DNA/protein absorbance ratio of 260nm/280nm. The DNA was stored at -20^{o}C until required.

2.4 Identification of Potential Sources of *M. kansasii* Infection

2.4.1 Collection and Processing of Water and Biofilm Samples

Water and biofilm samples were collected randomly from drinking water taps in mine hostels, change rooms, kitchens, boreholes, shower-heads, piped underground water and dams. The taps and showerheads were run for about 1min to clear the service lines prior to sample collection. At each collection site, water samples were collected in 1 litre sterile polyethylene bottles each containing 20mg of sodium thiosulphate to neutralise residual chlorine (Clesceri *et al*, 1999). Biofilms were collected by scraping interior surfaces of either taps or shower-heads with a sterile cotton-tipped swab and transferred to a 2ml solution of Ringer's solution (8.6g sodium chloride, 0.3g potassium chloride, and 0.33g calcium chloride per litre of water). A total of 27 water and 29 biofilm samples were obtained from the Vaal River gold-mining region, while 17 water and 15 biofilm samples were obtained from the Secunda coal-mining complex. Immediately after collection, all samples were placed in a cool box containing ice packs for transportation to the laboratory where they were stored at 4^{o}C until processed. The samples were processed within 48hrs of collection. Briefly, 1 litre of water was filtered through 0.45µl nitrocellulose membrane filters (Millipore Corporation, Bedford, MS, USA) by vacuum filtration using a Manifold Filtration System (Sartorius AG, Goettingen, Germany). The membranes were transferred into sterile screw-capped specimen containers with 10ml of 0.005% cetylpyridinium chloride (CPC) (Sigma-Aldrich, Steinheim, Germany) to reduce background organism levels. The surface of the membranes were abraded vigorously and thoroughly with a sterile platinum inoculating loop and cup-sonicated in a Brunson 1200 Sonifier (Brunson

Cleaning Equipment Company, Shelton, CT, USA) at 47kHz for 5min. Biofilm samples were first vortexed to dislodge bacteria from the swabs, centrifuged and the pellet resuspended in 10ml of 0.005% CPC. Both the water and biofilm samples were then exposed to the decontaminant at room temperature for 30min on a shaking platform. The decontaminated solution was then transferred into 15ml screw-capped tube, centrifuged at 3800rpm for 20min, the deposit washed twice with 1ml sterile normal saline and resuspended in 500µl of normal saline.

The choice and detection limit of the decontamination method for the isolation of *M. kansasii* from water was determined by comparing five of some of the commonly used decontamination methods. These included the use of 1% sodium hydroxide plus 3% sodium dodecyl sulphate, 2% NaOH plus 3% SDS, 3% NaOH plus 3% SDS and 4% NaOH plus 3% SDS and 0.005% CPC on a culture of *M. kansasii* ATCC 12478. One litre of sterile water was spiked with fresh liquid culture of *M. kansasii* to obtain final concentrations of 10^1, 10^2, 10^3, 10^4 and 10^5 colony forming units (CFU) per litre. The water samples were treated as described above. As compared to the other decontaminants, CPC was able to detect the presence of low levels of bacteria (10^2) and was thus selected as the ideal decontaminant for this work.

2.4.2 Isolation and Identification of Mycobacteria

One hundred microlitres of each of the resuspended decontaminated samples (from Section 2.2.1 above) were inoculated in triplicate on the surface of M7H10 agar medium (BD-Difco Laboratories, Sparks, MD, USA) supplemented with 10% OADC (Merck Chemicals, Darmstadt, Germany). The remaining pellets were used to make smears on microscope slides for acid-fast staining and for direct PCR screening of *M. kansasii*. The

plates were sealed with Parafilm (Pechiney Plastic Packaging Company, Chicago, IL, USA) and incubated at 25, 30 and 37^{o}C. The plates were examined every 2 days for the first 10 days and once a week thereafter for 2 months until considered negative. The number of colonies per litre or per swab of the original sample and colony type were noted. Single colonies of putative acid-fast bacteria, stained by the Ziehl-Neelsen (ZN) method, were picked and subcultured on LJ medium (Diagnostic Media Products, Johannesburg, South Africa). Identification to species level was done by biochemical tests, which included production of catalase, nitrate reductase, pyrazinamidase and Tween 80 hydrolysis tests (Leão *et al*, 2004) and by molecular methods described below.

The identity of *M. kansasii* clinical isolates was carried out and confirmed by West Vaal Hospital Tuberculosis Laboratory according to standard procedures such as morphology, growth rate, biochemical tests and by the use of the AccuProbe Assay (Gen-Probe, San Diego, CA, USA).

2.4.2.1 PCR Screening of *M. kansasii* isolates

In order to help in confirming the identity of *M. kansasii* strains obtained from both clinical and environmental sources, a *M. kansasii*-specific PCR identification method was employed, as it proved to be a rapid and cheaper alternative to the biochemical tests and the AccuProbe test. The test was performed on both decontaminated water and biofilm sample deposits and on isolated acid-fast colonies. This assay utilises primers, Fw1 (5'-CGGCCATTGTTCTACAGTCT-3') and Rv1 (5'-TAGAGATCCTCGCTTTGGT-3') that result in a 167bp PCR product. The primer sequences were freely accessed from http://www.freepatentsonline.com/ EP1881068.html ("Primer and probe for use in detection of *Mycobacterium kansasii* and method for detection of *Mycobacterium kansasii*

using the same"). Briefly, a loopful of mycobacterial growth was suspended in 400µl of TE-Triton X-100 buffer (10mM Tris, 1mM EDTA, pH 8.0, 1% Triton X-100) in a microcentrifuge tube and boiled for 15min. The lysate was subjected to three 10min cycles of freeze-thaw cycles, centrifuged for 5min at 10 000rpm and the supernatant subjected to PCR with the *M. kansasii*-specific primers. The amplification was achieved by the addition of 5µl of the supernatant DNA to a reaction mixture containing 200µM of each deoxynucleoside triphosphate (dNTP), 1µM of each primer, 1x Mastermix (50mM KCl, 10mM Tris-HCl [pH 8.3], 1.5mM $MgCl_2$, 0.001 [wt/vol] gelatine, and 1.25U of *Taq* polymerase [Fermentas Life Sciences, Glen Burnie, MD, USA]) in a total volume of 25µl. The reaction tube was first heated for 5min at 94°C and then subjected to 35 cycles of amplification for 1min at 94°C, 1min at 57°C, 1min at 72°C, followed by a 10min extension step at 72°C on an iCycler Thermocycler (Bio-Rad, Hercules, CA, USA). The presence of the 167bp amplicon was detected by electrophoresis of 5µl of the amplicon on a 1.5% SeaKem LE agarose gel (Lonza, Rockland, ME, USA). The specificity of these primers was assessed by including DNA samples from other mycobacterial strains: *M. gastri*, *M. tuberculosis*, *M. bovis*, *M. scrofulaceum*, *M. gordonae*, *M. avium*, *M. intracellulare*, *M. xenopi*, *M. fortuitum, M. abscessus, M. peregrinum* and *M. chelonae*. None of these mycobacterial species was PCR-positive with the *M. kansasii*-specific primers.

2.4.3.2 PCR-Restriction Analysis

Polymerase Chain Reaction-Restriction Analysis (PRA) was primarily employed to assist in the subtyping of *M. kansasii* isolates and was carried out as described previously (Telenti *et al*, 1993). The test was carried out in three stages: genomic DNA was prepared in the form of supernatants, subjected to PCR amplification by targeting the heat shock

protein 65 gene (*hsp65* gene) and the resulting amplicons analysed by restriction enzymes. The results of the restriction fragments were then evaluated with the help of a published algorithm (Devallois *et al*, 1997; Chimara *et al*, 2008) and compared to PRASITE (http://app.chuv.ch/parasite/index.html), an internet database maintained by the Swiss National Centre for Mycobacteria.

2.4.3.2.1 DNA Extraction and PCR Amplification of the *hsp65* Gene

Genomic DNA was prepared in the form of thermolysates as described under section 2.2.3.1 above. The *hsp65* gene was amplified from the cell supernatants by PCR using primers Tb11 (5'-ACC AAC GAT GGT GTG TCC AT-3') and Tb12 (5'-CTT GTC GAA CCG CAT ACC CT-3'), which result in a 441bp amplicon (Telenti *et al*, 1993).

The PCR was carried out in a final volume of 50µl consisting of 10µl of DNA and 40µl of PCR premixed solution containing 200µM of each dNTP, 1µM of each primer, 1x Mastermix (Fermentas Life Sciences, Glen Burnie, MD, USA). DNA from *M. kansasii* type strain ATCC 12478 was used as a positive control while deionised water was used as a negative control. The reaction tube was first heated for 10min at 94°C and then subjected to 45 cycles of amplification for 40s at 94°C, 50s at 60°C, 1min at 72°C, followed by a 10min extension step at 72°C on an iCycler Thermocycler (Bio-Rad, Hercules, CA, USA). The presence of the 441bp amplified product was detected by electrophoresis of 5µl of the amplicon on a 1.5% SeaKem LE agarose gel (Lonza, Rockland, ME, USA).

2.4.3.2.2 Enzyme Restriction and Analysis of the *hsp65* gene PCR products

Twenty microlitres of PCR products were digested separately with 10U of *Bst*EII and *Hae*III (Fermentas Life Technologies, Glen Burnie, MD, USA) in 1x restriction buffer

(Fermentas Life Sciences, Glen Burnie, MD, USA) in sterile deionised water. The reaction mixtures were incubated for 3hr at 60°C for the *Bst*EII digestions and at 37°C for the *Hae*III digestions. Restriction patterns were analysed by agarose gel electrophoresis. Briefly, samples were prepared for electrophoresis by adding 3µl of 6x gel loading buffer (Fermentas Life Sciences, Glen Burnie, MD, USA) to 22µl of the digest and loaded onto a 3% SeaKem LE agarose gel (Lonza, Rockland, ME, USA) containing 0.5µg/ml ethidium bromide. A 50bp marker (Fermentas Life Sciences, Glen Burnie, MD, USA) was loaded on opposite sides and centre of the gel and was used for sizing the resulting fragments. The gel was prepared and electrophoresed in 0.5x Tris-Borate-EDTA (TBE) electrophoresis buffer (10x buffer: 108g Tris, 55g boric acid, 9.3g EDTA in a litre of water [pH 8.0]) for 60 min at 100V, 300mA. After electrophoresis, the gels were imaged on a Gel Doc XR Documentation System (Bio-Rad Laboratories, Hercules, CA, USA). For the analysis and interpretation of the PRA patterns, Quantity One Software version 4.6.8 (Bio-Rad, Hercules, CA, USA) that converts the migration distance in millimetres to apparent molecular size in base pairs, was used. The results for the restriction fragments were then evaluated with the help of a published algorithm (Devallois *et al*, 1997; Chimara *et al*, 2008) and compared to PRASITE (http://app.chuv.ch/parasite/index.html), an internet database maintained by the Swiss National Centre for Mycobacteria. Any restriction fragment below 50bp was disregarded in order to avoid confusion with primer-dimer bands.

2.4.3.3 DNA Sequencing

To confirm the identity of the mycobacteria detected, DNA sequencing of the mycobacterial *hsp*65, s*ecA1*, *gyrB* genes or 16S-23S rDNA Internal Transcribed Sequence (ITS) was performed. The genes were first amplified by PCR in a final volume of 50µl

containing 10µl of mycobacterial thermolysate, prepared as described under Section 2.2.3.1 above, and 40µl PCR master mix: 200µM of each dNTP, 2µM of each primer (Tb11 and Tb12 for the *hsp*65 gene or ITS1, 5'-GATTGGGACGAAGTCGTAAC-3' and ITS2, 5'-AGCCTCCCACGTCCTTCATC for the ITS gene [Roth *et al*, 2000] or RPO5, 5´-TCAAGGAGAAGCGCTACGA-3´ and RPO3, 5´-GGATGTTGATCAGGGTCTGC-3´ for the *gyrB* gene [Lee *et al*, 2003] or MtuF, 5'-GACAGYGAGTGGATGGGYCGS GTGCACCG-3' and MtuR, 5'-ACCACGCCCAGCTTGTAGATCTCGTGCAGCTC-3' for the *secA1* gene [Zelazny *et al*, 2005]), 1x Mastermix (50mM KCl, 10mM Tris-HCl [pH 8.3], 1.5mM $MgCl_2$, 0.001 [wt/vol] gelatine, and 1.25U of *Taq* polymerase [Fermentas Life Sciences, Glen Burnie, MD, USA]). The reaction tube was first heated for 10min at 95°C and then subjected to 35 cycles of amplification for 1min at 95°C, 1min at 60°C (for the Tb and RPO primers) or 57°C (for the ITS primers) or 65°C (for Mtu primers), 1min at 72°C, followed by a 10min extension step at 72°C on an iCycler thermocycler (Bio-Rad, Hercules, CA, USA). The presence of the 480, 441, 700 and 360bp amplified products for the ITS sequence, *hsp*65, *secA1* and *gyrB* genes, respectively, was detected by electrophoresis of 5µl of the amplicons on a 1.5% SeaKem LE agarose gel (Lonza, Rockland, ME, USA).

Prior to sequencing, the PCR products were first purified from agarose gels with the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. Forward and reverse linear amplification was performed in 10µl using 2µl of the purified PCR product (about 20 to 200ng), 2µl BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), 1µl BigDye Sequencing Buffer (Applied Biosystems, Foster City, CA, USA) and 1µM of Tb11 or Tb12 or ITS1 or ITS2 or RPO5 or RPO3 or MtuF or MtuR primer. Linear amplification

consisted of 25 cycles of denaturation at 96°C for 10s, annealing at 60°C for 30s and elongation at 72°C for 60s using the iCycler Thermocycler (Bio-Rad, Hercules, CA, USA). Fluorescence-labelled DNA was purified using the ethanol precipitation method (Ausubel *et al*, 2001). Briefly, the entire extension products were transferred into 80µl of freshly prepared precipitation solution (3µl of 3M sodium acetate [pH 4.6], 62.5µl of non-denatured 95% ethanol and 14.5µl deionised water), incubated for at least 1hr at room temperature and centrifuged at 14000rpm for 20min. After carefully removing the supernatant, 250µl of 70% ethanol was added to the pellet, vortexed and the contents re-centrifuged at 14000rpm for 8min. The ethanol was carefully aspirated and the pellet air-dried for 15min at room temperature. The samples were analysed at the Inqaba Biotechnical Industries Sequencing Facility (Pretoria, South Africa) on an ABI PRISM 3730XL DNA analyser (Applied Biosystems, Foster City, CA, USA). The DNA sequence reads were edited using Ridom TraceEdit software (Ridom Bioinformatics GmbH, Würzburg, Germany) and used to search the National Center for Biotechnology Information (NCBI) RefSeq database using BLASTN software (http://www.ncbi.nlm.nih.gov/BLAST). A distance score of 0.00% to less than 1.00% was used as the criteria for species identity.

2.5 Determination of Genetic Diversity Amongst Clinical and Environmental Isolates of *M. kansasii* by Macrorestriction Analysis using PFGE

The *M. kansasii* clinical isolates were first subtyped by PRA of the *hsp65* gene as described in Section 2.4.3.2. Confirmation of *M. kansasii* isolates not showing the typical *M. kansasii* restriction patterns was carried out by DNA sequencing of the *secA1* and *gyrB* genes, and the ITS spacer region. The predominant subtype, subtype 1, was then subjected to further analysis by macrorestriction analysis as described below.

To assess the clonal relationships amongst the subtype I *M. kansasii* clinical and environmental isolates, an analysis of chromosomal DNA restriction patterns was performed by Pulsed-Field Gel Electrophoresis (PFGE) (Chapter 4). DNA for PFGE was prepared from stirred broth cultures as previously described (Hughes *et al*, 2001).

Figure 2.2. An IKAMAG (IKA-Werke GmbH & Co.KG, Staufen, Germany) multiple position magnetic stirrer used in the culture of *M. kansasii* isolates for PFGE analysis. Ten millilitre cultures, contained in universal containers, were stirred continuously using magnetic stirrer bars on an IKAMAG to prevent clumping (Adapted from Hughes *et al*, 2001).

2.5.1 Preparation of Agarose Plugs

Briefly, loopfuls of each isolate were inoculated into 10ml of Middlebrook 7H9 broth (BD-Difco Laboratories, Sparks, MD, USA) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) (Merck Chemicals, Darmstadt, Germany), 0.05% Tween 80 and 2.5% glycerol in 30ml sterile plastic disposable universal containers. After adding sterile magnetic stirring bars (12 x 6mm) to the containers and sealing them with parafilm, they were placed on a 15 Point IKAMAG Magnetic Stirrer (IKA-Werke GmbH & Co.KG, Staufen, Germany) (Figure 2.2) and incubated at 37^{o}C for about 7 days or until turbid (OD_{550nm} 2.0). Two millilitres of cells were harvested at room temperature by centrifugation at 3800rpm for 20min and resuspended in 500µl spheroplasting buffer (20mM citrate phosphate buffer, pH 5.6 [0.2M citric acid, 0.5M disodium hydrogen

phosphate], 50mM EDTA and 0.1% (w/v) Tween-80). After warming the cell suspension to 55^{o}C for 15min in a waterbath, an equal volume (500µl) of 1.5% low melting agarose (Bio-Rad Laboratories, Hercules, CA, USA) in 50mM EDTA was added and the mixture was then poured into pre-cooled moulds (Bio-Rad Laboratories, Hercules, CA, USA) and allowed to set for approximately 20 to 30min. The cells in the agarose plugs were incubated in freshly prepared Tris-EDTA (TE) buffer (pH 8.0) containing 4mg/ml of lysozyme (Sigma-Aldrich, Steinheim, Germany) for at least 48hrs at 37°C and then in freshly prepared ESP solution (2mg/ml proteinase K and 1% N-lauryol sarcosine [Sigma-Aldrich, Steinheim, Germany] in 0.5M EDTA, pH 8.0) for 7 days at 55°C. At the end of the incubation period, the plugs were topped up with 2ml 0.5M EDTA, sealed with parafilm and stored at 4^{o}C until required.

2.5.2 DNA Quality Check

Plugs were gently transferred to a Petri dish and 3mm sections were cut with a scalpel blade. The plug sections were transferred into microcentrifuge tubes and gently washed 3 times (10min each) in 1ml TE buffer on a shaking platform. The plugs were then transferred to other tubes with as little TE buffer as possible. The remaining TE buffer was spun down, removed with a pipette and the tube placed in a 70^{o}C waterbath for approximately 1min to melt the sections. The sample was mixed thoroughly and 30µl loaded onto a 1% agarose mini gel in 0.5x TBE. The gel was electrophoresed at 70V for approximately 30min. Upon examination, the intact DNA appeared as a distinct band close to the wells while degraded DNA appeared as a smear.

2.5.3 Restriction Endonuclease Digestion

Slices 5mm thick were cut from the plugs in Petri dish, washed 7 times with 1hr changes in TE buffer (pH 8.0), pre-equilibrated in 300µl of 1x restriction buffer containing 0.1mg/ml BSA (Fermentas Life Sciences, Glen Burnie, MD, USA) for 1hr at room temperature and then incubated in 100µl of 1x restriction buffer containing 0.1mg/ml BSA and 30U *Dra*I (Fermentas Life Sciences, Glen Burnie, MD, USA). After overnight incubation at 37°C, fresh restriction reaction mixture was added and incubated for a further 4 to 6hrs to ensure complete digestion of the samples.

2.5.4. Electrophoresis

The restricted sample was loaded onto a 1% (wt/vol) low-meting point agarose gel (Bio-Rad Laboratories, Hercules, CA, USA) in 0.5x TBE (10x stock: 89mM Tris, 89mM boric acid, 2mM EDTA [pH 8.0]), sealed with 1% agarose in 0.5x TBE and allowed to set. PFG MidRange II DNA markers (New England BioLabs, Ipswich, USA) were loaded onto gels as molecular size standards and the gel was pre-equilibrated for 1hr at 14°C in a CHEF-DRIII electrophoretic tank (Bio-Rad Laboratories, Hercules, CA, USA) filled with 2 litres of 0.5x TBE. The gel was then run for 40hrs at 14^{o}C using the following parameters: angle, 120°; gradient, 6v/cm; pump setting, 70pmb; ramp, linear; and switch times, 6.75 to 35.38s. The parameters were selected to separate fragments in the range 30 to 400kb. Following electrophoresis, the gels were stained with 0.5µg/ml ethidium bromide for 30min, and after destaining for 30min, the gels were imaged with a Gel Doc XR Documentation System (Bio-Rad Laboratories, Hercules, CA, USA).

2.5.5 Macrorestriction Pattern Analysis

A TIFF image of each gel was exported to the Gel Compar 6.0 System (Applied Maths, Kortrijk, Belgium) for analysis. A PFG MidRange II DNA marker (New England BioLabs, Ipswich, Glen Burnie, USA) was included on each gel to facilitate molecular weight assignments and inter-gel comparisons. For inter-gel comparisons, the two outermost and centre lanes of each gel were used.

2.6 Identification of Genetic Differences Between Clinical and Environmental Isolates of *M. kansasii*

Hybridisation-Monitored Genome Differential Analysis (HMDA) was used to directly detect and clone genomic differences between *M. kansasii* subtype I (tester) and the other selected *M. kansasii* subtypes III, V and VI (driver) with a view to identifying unique or divergent sequences found in subtype I. HMDA is a PCR-based solid subtractive hybridisation method that utilises a ribosomal DNA (rDNA) monitoring system to tract the entire subtraction process between two DNA populations (Yueqing *et al*, 2006). The selection of *M. kansasii* subtype I as the target strain was based on the fact that it is the most frequently isolated subtype from human clinical samples and is generally considered to be pathogenic as compared to the other subtypes (Zhang *et al*, 2004; Goh *et al*, 2006).

2.6.1 Preparation of Tester DNA

2.6.1.1 Restriction of Tester DNA

M. kansasii subtype I clinical isolate genomic DNA was used as the tester DNA in the subtraction process. To prepare the tester DNA for hybridisation, the DNA was digested with T*sp*509I (New England BioLabs, Ipswich, MA) as previously described (Yueqing *et al*, 2006). *Tsp*509I creates 5'-AATT overhangs on genomic DNA fragments that allow

subsequent ligation to compatible ends, in this case an adapter molecule. Four micrograms of genomic DNA was digested in total volume of 50µl containing 10U of *Tsp*509I and 1x *Tsp*509I restriction buffer 1 (New England BioLabs, Ipswich, MA, USA) in deionised water. The reaction was incubated at 65^{o}C for 4hr. The efficiency of digestion was assessed by running 5µl of the digest mixture on a 1% gel.

2.6.1.2 Purification of Tester DNA

The digested DNA was then purified using the phenol-chloroform method. Briefly, 50µl of phenol/chloroform/isoamyl alcohol (24:24:1) (Merck Chemicals, Darmstadt, Germany) were then added, vortexed and centrifuged for 10 min at 14 000rpm at room temperature. The aqueous supernatant was carefully collected and transferred to a fresh tube. Fifty microlitres of chloroform/isoamyl alcohol (24:1) (Merck Chemicals, Darmstadt, Germany) were then added, vortexed and centrifuged for 5 min at 14 000rpm. The chloroform/isoamyl extraction process was repeated. The resulting aqueous supernatant was transferred to a fresh tube and 0.5 volume of 4M ammonium acetate and 2.5 (of the resulting volume) of 95% ethanol were then added to precipitate the DNA. After thoroughly vortexing and centrifugation at 14 000rpm for 20min, the pellet was washed once for 8min at 14 000rpm with 200µl of 70% ethanol, air-dried and dissolved in 30µl of 1x TE buffer . The DNA was stored at -20^{o}C until required.

2.6.1.3 Preparation of Adaptor DNA

The next step in the preparation of the tester DNA involved ligation of a phosphorylated adaptor to the digested tester (*M. kansasii* subtype I) DNA. This adaptor molecule was used as a target for PCR amplification of the tester DNA. Two oligonucleotides, 5'-CGG GTA CCG AGC TCG-3' and 5'-AAT TCG AGC TCG GTA CCC G-3' (phosphorylated

at the 5' end) (Yueqing *et al*, 2006), from a commercial source were annealed as follows: 40µl of each of the 5µM oligonucleotides (to give a final concentration of 2µM) were mixed in a microcentrifuge tube containing 10µl annealing buffer (10mM Tris, pH 8.0, 50mM NaCl, 1mM EDTA) and 10µl deionised water. The oligonucleotides were then boiled in a beaker of water for 5min and allowed to cool slowly to room temperature to permit them to anneal and avoid single-stranded oligonucleotides to self-anneal.

2.6.1.4 Ligation of Adaptors to Tester DNA

One hundred to five hundred nanograms of tester DNA were mixed with 5µl of the annealed phosphorylated linkers in tube containing 2U of T4 DNA ligase (0.4µl) and 1x ligation buffer (Promega, Madison, WI, USA) in total volume of 20µl. The tube contents were briefly vortexed, spun for 3-5s at high speed and incubated overnight at 4°C. Inactivation of the DNA ligase was achieved at 65°C for 10min. The ligation mixture was then used in the hybridisation experiment.

2.6.1.5 PCR Verification of Tester-Adaptor Ligation

This was performed to verify the presence or absence of the ligated adaptors on the tester genomic DNA using an adaptor-specific primer P1 primer (5'-CGG GTA CCG AGC TCG AAT T-3') (Yueqing *et al*, 2006). This PCR amplification step was also used for selecting tester DNA-specific sequences after hybridisation with the Driver genomic DNA. The amplification of the tester DNA was achieved by the addition of 100 to 500ng of the template DNA to a reaction mix containing 10mM potassium chloride, 50mM Tris-HCl, pH8.3, 2U FastStart Taq DNA polymerase (Roche Applied Science, Mannheim, Germany), 1x GC-rich solution, 5mM ammonium sulphate, 200µM of each dNTP, 2mM magnesium chloride, 1µM P1 primer in a total volume of 25µl. Thermal cycling was

performed on a Bio-Rad iCycler thermocycler using a programme consisting of an initial melting phase of 5 min at 94°C, followed by 25 cycles of a 30s melt at 94°C, annealing of 30s at 60°C and extension of 2min at 72°C, then a final extension hold for 10min at 72°C.

2.6.2 Preparation of Driver (Blocking) DNA

M. kansasii subtypes III, V and VI were selected and used for the preparation of driver genomic DNA. About 30µg/ml of the DNA was fragmented by heating to 95°C on a Bio-Rad iCycler thermocycler. Briefly, the DNA in 100µl of TE buffer was subjected to heat at 95°C using a time series of 5 to 45min to generate fragments between 1 to 5kb. The size of the fragments was ascertained on a 1% agarose gel. The DNA fragments were precipitated with equal volumes of 4M ammonium acetate and 100% ethanol, and dissolved in 30µl TE buffer (Shanks *et al*, 2006a). The DNA was then denatured with 4mM sodium hydroxide and 100mM EDTA, to a final concentration of 0.4mM and 10mM, respectively, and incubated for 5min at room temperature. Two microlitres of the denatured driver DNA were transferred onto each side of a 6mm diameter Hybond N+ nylon membrane disc (Amersham Biosciences, Buckinghamshire, UK) pre-wetted with distilled water. The wet discs were exposed to ultraviolet (UV) radiation for 3 to 5min to allow the DNA to bind covalently to the membrane. The discs were then baked for 1hr at 80^{o}C. The discs were rinsed twice for 1 hr (30 min x 2) with 5x SSC and twice again with 2x SSC (30 min x 2) at 60^{o}C. The washings were retained for detection of rDNA by PCR. This helped to confirm whether or not the driver DNA was removed from the discs. The quantity of driver DNA immobilised on each disc was the difference between the amount of DNA initially loaded onto the disc and the amount found eluted during the washing process (Yueqing *et al*, 2006). The discs were dried, sealed and store at 4^{o}C until required.

2.6.3 Subtractive Hybridisation and Monitoring

The methodology for subtractive hybridisation was performed as previously described with some modifications (Yueqing *et al*, 2006). The tester DNA, in 5x SSC buffer, was first denatured by heating to 95^{o}C for 5min and rapidly cooling on ice. Hundred microlitres of the denatured DNA were transferred to a 96-well flat-bottomed microtitre plate containing a nylon disc with immobilised driver DNA. Adjacent wells were filled with distilled water to avoid evaporation of the hybridisation buffer. The hybridisation process was carried out on an MS1 Microtitre Plate Minishaker (400rpm) (IKA Works Inc, Wilmington, NC, USA) at 60^{o}C for 24 to 30hrs. Ten microlitre samples were collected after every 4hrs for 16S rDNA analysis and the hybridisation process repeated by heat-denaturing the liquid phase containing the tester and by adding the liquid to a new well with a new disc. The hybridisation process was monitored by PCR analysis of the partial sequence of the 16S rDNA of the tester using primer pairs BC70 and BC71. The PCR was carried out in a total volume of 25µl containing 200µM of each dNTP, 2µM of each primer (BC70, 5'-TAACACATGCAAGTCGAACG-3' and BC71, 5'-CGTATTACCGCGGCTGCTGG-3' for the 16S rRNA gene, 1x Mastermix (50mM KCl, 10mM Tris-HCl [pH 8.3], 1.5mM $MgCl_2$, 0.001 [wt/vol] gelatine, and 1.25U of *Taq* polymerase [Fermentas Life Sciences, Glen Burnie, MD, USA]). The reaction tube was first heated for 10min at 95°C and then subjected to 35 cycles of amplification for 1min at 95°C, 1min at 61°C, 1min at 72°C, followed by a 10min extension step at 72°C on an iCycler thermocycler (Bio-Rad, Hercules, CA, USA). The presence of the 475bp was detected by electrophoresis of 5µl of the amplicons on a 1.5% SeaKem LE agarose gel (Lonza, Rockland, ME, USA).

After completion of subtraction of homologous sequences, a minimum of five P1 single primer PCR amplifications in 50µl volumes, as described under Section 2.5.1.5 were carried out using the FastStart High Fidelity Taq DNA polymerase (Roche Applied Science, Mannheim, Germany). After pooling the PCR products, the amplicons were then purified using a QIAquick PCR Product Clean-up Kit (Qiagen GmbH, Mannheim, Germany) according to the manufacturer's instructions.

2.6.4 Cloning of Enriched Tester PCR Products

The purified PCR products were ligated into a pGEM T-Easy vector (Promega, Madison, WI, USA). The purified insert and vector were mixed at a ratio of 3:1, respectively, and ligated overnight at 4^{o}C in a total volume of 10µl containing 1U of T4 DNA ligase and 1x ligase buffer (Promega, Madison, WI, USA). A ligation control consisting of a linearised vector was also set up to monitor the reaction. Inactivation of the DNA ligase was performed at 65^{o}C for 15min. The ligation products were then used to transform competent DH5α *E. coli* cells (Invitrogen Life Technologies, Paisley, UK).

2.6.5 Transformation of Competent Cells

Fifty microlitres of DH5α *E. coli* competent cells (Invitrogen Life Technologies, Paisley, UK) were gently mixed with 2µl of the ligation mix in a pre-cooled microcentrifuge tube by flicking the bottom of the tube and left on ice for 30min. The cells were then subjected to heat shock at 42°C without shaking for 30s and immediately transferred to ice for 2min. Phenotypic expression of the cells was allowed by the addition of 250µl of room temperature antibiotic-free SOC (Super Optimal Catabolite repression) medium (2.0g tryptone, 0.5g yeast extract, 1ml 1M NaCl, 0.25ml 1M KCl, 1ml 2M Mg^{2+} stock, 1ml 2M glucose up to 100ml, final pH 7.0) (Invitrogen Life Technologies, Paisley, UK),

incubating and shaking the tubes horizontally at 150rpm for at least 1hr at 37°C. The transformed cells were then selected by plating 50µl aliquots of each transformation reaction onto pre-warmed duplicate Luria-Bertani (LB) agar plates (Sigma-Aldrich, Steinheim, Germany) containing 100µg/ml ampicillin (Sigma-Aldrich, Steinheim, Germany), 100µl 100mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 50µl 20mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Promega, Madison, WI, USA). The plates were incubated overnight at 37°C and examined for growth of white and blue colonies. White colonies were further analysed as these generally contain inserts.

2.6.6 Screening of Cloned DNA Fragments

2.6.6.1 PCR Screening

In order to detect the presence of the cloned PCR inserts in the vector, PCR was directly performed on lysed bacterial colonies using the P1 primer (sequence information under Section 2.6.1.5). Briefly, template DNA was prepared by picking fresh single bacterial colonies from the LB plates with sterile tooth picks and suspending them in 50µl of sterile distilled water in microcentrifuge tubes. The suspension was boiled for 15min, centrifuged and 5µl of the resulting supernatant was used in the PCR reaction. The PCR conditions were set as described under Section 2.6.1.5.

2.6.6.2 Enzymatic Analysis

A single colony of the DH5α *E. coli* cells containing the pGEM T-Easy plasmid with the insert was used to inoculate 3ml of LB broth (Sigma-Aldrich, Steinheim, Germany) containing 100µg/ml ampicillin (Sigma-Aldrich, Steinheim, Germany) and grown in a shaking incubator overnight at 37^{o}C. After centrifuging the cells at 3800rpm for 15min, the plasmid DNA was prepared using the Qiagen Spin Plasmid Miniprep Kit (Qiagen

GmbH, Mannheim, Germany) according to the manufacturer's instructions. The DNA concentration for each sample was estimated at A_{260nm} in a spectrophotometer and stored at 20^{o}C until required.

Plasmid DNA from PCR positive clones were further analysed for the presence of the tester DNA inserts by *Eco*RI digestion. Briefly, plasmid DNA was digested in a total volume of 20µl containing 2µl DNA, 1x restriction buffer and 10U of *Eco*RI (Promega, Madison, WI, USA). After incubation for 2hrs at 37^{o}C, the restriction fragments were separated on a 1% agarose gel and photographed.

2.6.6.3 Dot Blot Hybridisation Analysis

To determine the efficiency of the subtraction process, dot blot analysis was carried out using the subtracted tester DNA and driver DNA as probes.

2.6.6.3.1 Probe Preparation

Tester and driver DNA was prepared as described under Sections 2.5.1 and 2.5.2, respectively. For the preparation of the driver probe, a defined terminal sequence was first added to the fragmented genomic DNA. Briefly, about 10µl (about 100ng) of DNA in a microcentrifuge tube were boiled for 10min in beaker of water in the presence of 125µM K9 DNA primer (5'-GACACTCTCGAGACATCACCGGTACCNNNNNNNNN-3') (Grothues *et al*, 1993; Shanks *et al*, 2006a) and 5µl of 10X Klenow buffer (Fermentas Life Sciences, Glen Burnie, MD, USA) in a total volume of 40µl. After cooling the tube on ice, the DNA was centrifuged briefly and 5U of DNA polymerase Klenow I fragment (Fermentas Life Sciences, Glen Burnie, MD, USA) and 3µl (0.33mM) dNTPs were added and the volume raised to 50µl with distilled water. The mixture was incubated at 37^{o}C for

2hrs and the reaction stopped by heating at 75^{o}C for 10min. The labelled DNA fragments were then purified with the QIAquick PCR Product Clean-Up Kit (Qiagen GmbH, Hilden, Germany).

For tester DNA probe synthesis, the DNA was amplified using the P1 primer as it contained the attachment site for the primer (refer to Section 2.5.1.5) while driver DNA was amplified by the K9 PCR primer (5'-GACACTCTCGAGACATCACCGG-3' (Grothues *et al*, 1993; Shanks *et al*, 2006a) using the Digoxigenin (DIG) Labelling Kit (Roche Applied Sciences, Mannheim, Germany) according to the manufacturer's instructions. The probes were then purified with a QIAquick PCR Product Clean-Up Kit (Qiagen GmbH, Hilden, Germany). Approximately 1µg of the probe was added to the hybridisation solution (5x SSC buffer, 0.1% N-lauroyl sarcosine, 1% blocking reagent [skimmed milk powder dissolved in maleic acid buffer {0.1M maleic acid and 0.15M sodium chloride, pH 7.5}], and 0.02% SDS) and stored at -20^{o}C until required.

2.6.6.3.2 Dot Blotting

For dot blot analysis, inserts from positive clones were first amplified with the P1 primer. The PCR products were purified with the QIAquick PCR Product Clean-Up Kit (Qiagen GmbH, Hilden, Germany) and 10µl resuspended in 45µl of denaturation solution (0.5M sodium hydroxide, 1.5M sodium chloride). The denatured DNA from each clone was then applied to a separate well of a 48-well dot blot apparatus (Bio-Rad Laboratories, Hercules, CA, USA) and transferred under vacuum to a Hybond N^{+} membrane (Amersham Biosciences, Buckinghamshire, UK) which had been pre-equilibrated in 6x SSC buffer (prepared from 20x SSC buffer [3M sodium chloride, 0.3M sodium citrate, pH 7.0). The DNA was then neutralised with 20µl neutralisation solution (1M Tris-HCl, pH 8.0, 1.5M

sodium chloride), washed with 2x SSC buffer, the membrane air-dried and the DNA fixed by exposure to ultraviolet light for 3min on a transilluminator (Spectroline Corporation, New York, USA). Two membranes were prepared, one for probing with the tester probe and the other one for probing with the driver probe.

2.6.6.3.3 DNA Hybridisation and Detection

The nylon membranes were inserted into Thermo-Hybaid HB-OV-BS tubes (Thermo Hybaid, Middlesex, UK) containing 50ml pre-hybridisation solution (5x SSC buffer, 0.1% N-lauroyl sarcosine, 1% blocking reagent and 0.02% SDS) and pre-hybridised at 65^{o}C for 1.5hrs in a Mini Hybridisation Oven MK II (MWG-Biotech AG, Ebersberg, Germany). The DIG-labelled probes were denatured by boiling in water for 5min, rapidly chilled on ice, added to the hybridisation tubes to replace the prehybridisation solution, and left to hybridise to the immobilised DNA overnight at 65^{o}C. The probes were carefully decanted into 15ml tubes and stored at -20^{o}C for re-use. The membranes were first washed twice (15min each time) at room temperature on a mechanical shaker in a solution containing 2x SSC and 0.1% SDS, and twice again at 68^{o}C with washing solution (0.5x SSC and 0.1% SDS) in hybridisation tubes for 15min each time. The membranes were then removed from hybridisation tubes, equilibrated in 1x washing buffer (0.3% Tween 20 in maleic acid buffer [0.1M maleic acid, 0.15M NaCl, pH 7.5]) for 1min at room temperature and blocked in 1x blocking solution (prepared from a 10x solution in maleic acid buffer) on a mechanical shaker for 30min at room temperature. The membranes were stained with anti-digoxigenin-alkaline phosphatase antibody (Roche Applied Science, Mannheim, Germany), diluted 1:10 000 in 1x blocking reagent, for 30min at room temperature on a mechanical shaker. After washing twice with the wash buffer (15min each time) the membranes were equilibrated in detection buffer (100mM Tris-HCl, pH9.5, 100mM

sodium chloride) for 2min and sealed in a hybridisation plastic bag using leaving one side unsealed. Three millilitres of freshly prepared CSPD (sodium 3-{4-meth-oxyspiro[1,2-dioxetane-2,2'-(5'-chloro) tricycle[3.3.1.13,7] decan phenyl phosphate) (Roche Applied Science, Mannheim, Germany) substrate solution (diluted 1:100 in 1x detection buffer) were added to each membrane, air bubbles removed and the remaining side of the bag sealed. The membranes were then incubated for 15min at room temperature. The substrate was expelled from the bags by rolling out the liquid using a disposable 10ml serological pipette through a hole cut at the top right hand corner of the bag. The bag was heat-sealed, wiped, transferred into a 24 x 30cm film cassette (Amersham Biosciences, Buckinghamshire, UK) and exposed to a high performance chemiluminescence film (Amersham Biosciences, Buckinghamshire, UK) at room temperature for about 15min in a dark room. The film was processed on a Kodak RPX-OXMAT Processor (Eastman Kodak Company, Rochester, New York, USA) at the Radiology Department of the Charlotte Maxeke Johannesburg Academic Hospital.

2.6.7 DNA Sequencing

Cloned inserts showing hybridisation to the tester, and not to the driver probe, were sequenced using T7 primers (Promega, Madison, WI, USA). Briefly, plasmid DNA from PCR positive clones was prepared using the Qiagen Spin Plasmid Miniprep Kit (Qiagen GmbH, Hilden, Germany) as stated above. Forward linear amplification was performed in 10µl using 2µl of the purified plasmid DNA (about 20 to 200ng), 2µl BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK), 1µl BigDye Sequencing Buffer and 1µM of T7 primer (Promega, Madison, WI, USA). Linear amplification consisted of 25 cycles of denaturation at 96^{o}C for 30s, annealing at 50^{o}C for 30s and elongation at 72^{o}C for 4min using the Bio-Rad iCycler (Bio-Rad Laboratories,

Hercules, CA, USA). Fluorescence-labelled DNA was purified using the ethanol precipitation described under Section 2.2.3.3 and analysed on an ABI PRISM 3730XL DNA analyser (Applied Biosystems, Foster City, CA, USA) by Inqaba Biotechnical Industries (Pretoria, South Africa).

2.6.8 Sequence Analysis

DNA sequences were edited with VecScreen Software (http://www.ncbi.nlm.nih.gov /VecScreen/VecScreen.html) and Ridom TraceEdit (Ridom Bioinformatics GmbH, Würzburg, Germany), and used to search the National Centre for Biotechnology Information (NCBI) database using the Protein Clusters Database (ProtClustDB) with BLASTX software (http://www.ncbi.nlm.nih.gov/ BLAST) (Altschul *et al*, 1997). The sequences were also compared to the *M. kansasii* ATCC 12478 genome draft assembly consisting of 299 major contigs and 6.4 Mb using the NCBI BLASTX (http://www.ncbi.nlm.nih.gov//genomes/ geblast.cgi? gi=6107). BLASTX sequence matches with *E* (expectation) values of $\leq 10^{-3}$ and sequence identities of $\geq 60\%$ were regarded to be similar protein sequences (Pearl *et al*, 2000; Shanks *et al*, 2006b). DNA sequences were organised into functional gene categories using the Protein Clusters Database (ProtClustDB) (http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein clusters) (last accessed April 6, 2010) which contains both curated and uncurated clusters of proteins grouped by sequence similarity (Klimke *et al*, 2009).

2.7 Determination of Immunomodulatory Ability of *M. kansasii* Lymphocyte *In Vitro*

3.7.1 Preparation of Mycobacterial Cell Lysates

Mycobacterial cell lysates were used for pulsing human mononuclear cells. Mycobacteria were either obtained from LJ slopes by scraping colonies and suspending them in normal

saline or from broth cultures. The organisms were heat-killed, centrifuged at 3800rpm for 10min in a standard bench-top microcentrifuge to remove any traces of medium, washed three times with normal saline at 3800rpm for 30min and sonicated at 4^{o}C to disrupt the cells. Protein concentrations of the bacterial cell lysates were determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The lysates were then aliquoted and stored at -20^{o}C until required.

2.7.2 Preparation of Mononuclear Cells

Venous blood samples from normal healthy volunteers were collected in preservative-free heparin tubes (BD Biosciences, Plymouth, UK). Mononuclear (MN) cells were separated at room temperature by centrifugation for 40min at 4000rpm on a Hypaque-Ficoll (Sigma-Aldrich, Steinheim, Germany) gradient and collected from the plasma-Ficoll interface. The cells were washed 3 times in normal saline by centrifugation at 1200rpm for 7min and resuspended at various concentrations in RPMI-1640 with L-glutamine (Lonza, Wilkerville, MD, USA) containing 10% heat-inactivated foetal calf serum and 1% penicillin/streptomycin, depending on the experimental requirements.

2.7.3 Production of Suppressor Cell Activating Factors

It has been previously demonstrated that when macrophages ingest mycobacteria from their environment, they release bacterial components rich in lipids. These lipids have been termed Suppressor Cell Activating Factors (SCAF), and they have a suppressive effect on lymphocyte proliferation (Wadee *et al*, 1983). In this study SCAF was produced from U937 cells, a human monocytic cell line (courtesy of Professor Mary Gulumian, National Institute of Occupational Health, Johannesburg, South Africa). The cells were first grown

at 3 x10^6 cells/ml in RPMI-1640 at 37^{o}C in 5% CO_2 for 2hrs in the presence of 60µg/ml of protein from each of the mycobacterial lysates (*M. kansasii* subtypes I-VI, *M. tuberculosis*) in sterile 24-well plates (Becton-Dickinson and Company, New Jersey, USA) to stimulate the production of SCAF (Wadee *et al*, 1983). After 2hrs, the medium was discarded and the cells were washed 3 times at 1200rpm for 7min and resuspended in fresh RPMI-1640 medium to their original volume. The cultures were incubated for an additional 48hrs, after which time the supernatants were collected and stored at -20^{o}C until required. Control supernatants were prepared by incubating monocytes alone without the mycobacterial lysates.

2.7.4 Effect of SCAF on Lymphocyte Blastogenesis

To determine whether *M. kansasii* has the ability to induce a suppressive effect on lymphocyte proliferation, a lymphocyte transformation assay was performed. MN cells (n=5 healthy donors), at 2 x 10^5cell/ml, were cultured in 96-well round-bottomed microtitre plates (Nunc A/S Roskilde, Denmark) in the presence of an equal volume of SCAF. Cells were stimulated with 40µg/ml of concanavalin A (Pharmacia Biotech, Upsalla, Sweden). All experiments were performed in triplicate. Plates were incubated at 37^{o}C in 5% CO_2 in humidified air for 72hrs. Tritiated thymidine ([methyl-^{3}H]thymidine, 24 Ci/mmol) (Amersham, Buckinghamshire, England) at 1µCi, was added for the final 18hrs of culture. The cells were harvested onto glass fibre filters (Packard Instruments BV Chemical Operations, Groningen, The Netherlands) by using an automated FilterMate Cell Harvester (Perkin Elmer) and transferred into vials containing Insta-Gel II Universal Scintillation liquid (Packard Instruments BV Chemical Operations, Groningen, The Netherlands). Radioactivity was counted by liquid scintillation on a 1900TR Liquid Scintillation Counter (Packard Instrument Company Inc, Downers Grove, Illinois, USA).

Lymphocyte proliferation was expressed as the mean of counts per minute of radioactivity incorporated by triplicate cultures of 2 x 10^5cell/ml.

2.7.5 Effect of SCAF on Lymphocyte Activation

Previous studies have demonstrated that SCAF induced CD8+ T cells produce Suppressor Carbohydrates (SC) in response to *M. tuberculosis*, which had a modulatory effect on CD4+ T cells (Wadee *et al*, 1983; Sussman and Wadee, 1991). Thus, SC was found to induce a Th2 response rather than a protective Th1 response in human mononuclear (MN) cells. In this study we investigated whether SCAF from *M. kansasii* would modulate the activity of lymphocytes in the MN cells cell population through cytokine production or suppression. To establish the longitudinal profile of cytokine expression by MN cells pulsed with various mycobacterial preparations, cytokine expressions were measured at three different time points, i.e., 24, 48 and 72hrs. The 72hr time point was found to be the suitable optimal time point for the analysis.

2.7.5.1 Stimulation of MN Cells

MN cells at a concentration of 2 x 10^6cell/ml were cultured in 24-well plates (Nunc A/S Roskld, Denmark) in the presence of an equal volume of SCAF (*M. kansasii* subtypes I-VI) or positive control SCAF (*M. tuberculosis* H37Rv) or negative control SCAF (medium only) at 37^{o}C in 5% CO_2 in humidified air for 4hrs. The cells were stimulated with 12.5µg/ml of Phytohaemagglutinin (PHA) (Sigma-Aldrich, Steinheim, Germany). A positive control with only PHA, but no SCAF added and a non-stimulated negative control with medium only were also prepared.

2.7.5.2 Cytokine Analysis

To evaluate the levels of cytokines released by T cells in the presence of SCAF, the BD CBA Human Th1/Th2/Th17 Kit (BD Biosciences, San Jose, CA, USA) was used according to manufacturer's instructions. The kit is used to measure Interleukin-2 (IL-2), IL-4, IL-6, IL-10, Tumour Necrosis Factor-α (TNF-α), Interferon-γ (IFN-γ) and IL-17A protein levels in a single sample. MN cells (n=7 healthy donors), at 2 x 10^6cells/ml, were exposed to SCAF supernatants for 4hrs after which the supernatant was harvested at 12000rpm for 7 min.

The cells were then be resuspended in fresh RPMI-1640 to their original volume. The cultures were incubated for an additional 72hrs, after which the supernatants were collected. Fifty microlitres of supernatant were then used in the assay. The assay was performed according to the manufacturer's instructions and analysed on a FACS Calibur Flow Cytometer (BD Biosciences, San Jose, CA, USA).

2.8 Statistical Analysis

Data analysis was performed with GraphPad Prism Software Version 5.0 for Windows (GraphPad Software, San Diego, California, USA). Results were expressed as medians± SEM. Multiple comparisons were done with the Kruskal-Wallis test, followed by the Dunn's multiple comparison adjustment test. Tests were interpreted at 5% significance level (two-sided). For cytokine expression, all values were calculated with regard to the negative control in each assay (MN cells + PHA + untreated culture supernatant). *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with the negative control.

2.9 Ethics Approval

Ethics approval for this study was granted by the University of the Witwatersrand Human Research (Medical) Ethics Committee. The Ethics Clearance Certificate Number was M050707 (Appendix I). Permission to collect the environmental samples from the Vaal River region and *M. kansasii* clinical isolates was obtained from the Mine Health and Safety Council of South Africa, the AngloGold Ashanti Health Services, South Africa. Permission for environmental samples from the Secunda coal-mining complex was obtained from the Secunda Collieries, South Africa.

CHAPTER 3

Identification of Potential Environmental Sources of *M. kansasii* Infection

CHAPTER 3

Identification of Potential Environmental Sources of *M. kansasii* Infection

3.0 Abstract

M. kansasii is a major cause of NTM pulmonary disease in South African gold miners, but the source of infection is unknown. Since there is no evidence of person-to-person transmission and the organism has typically been isolated from water in other settings, the environment is considered the most likely source of infection. This study examined the presence of *M. kansasii* in water distribution systems supplying mines. Five *M. kansasii* strains, as well as 10 other potentially pathogenic mycobacteria, were isolated from biofilm and water samples obtained from showerheads and water taps. PCR-restriction analysis (PRA) of the 5 *M. kansasii* isolates revealed three subtype I strains, one subtype IV strain and a unique isolate that did not show a typical *M. kansasii* PRA pattern. The unique isolate may probably represent a new subtype of *M. kansasii*. Macrorestriction analysis, using PFGE, of the three subtype I isolates demonstrated that they were at least 85.6% similar to a set of clinical isolates. One of the significant findings was the observation that one of the three subtype I isolates was identical to a clinical isolate, suggesting that water, particularly from showerheads, is a potential source of *M. kansasii* infection for the miners and that water may present a significant health risk associated with this organism and other opportunistic pathogenic mycobacteria for the miners.

3.1 Introduction

A number of NTM infections have been attributed to contaminated water, and most of these NTMs have been isolated from public water distribution systems and natural water bodies (Peters *et al*, 1995; Kubalek and Mysak, 1996; Falkinham *et al*, 2001; Le Dantec *et al*, 2002; September *et al*, 2004; Vaerewijck *et al*, 2005; Falkinham *et al*, 2008). This suggests that water may be the vehicle by which these organisms infect or colonise humans. Exposure to mycobacteria in water may occur by drinking and by inhalation of aerosols generated through swimming, bathing and showering (O'Brien *et al*, 2000; Glazer *et al*, 2007; Feazel *et al*, 2009). Most of the NTMs responsible for these diseases are highly resistant to common disinfectants and other forms of treatment such as heat, which allow them to persist for long periods of time in water (Taylor *et al*, 2000; Bagh *et al*, 2004). Another factor in the survival of mycobacteria in water is their association with free-living amoebae and other protozoa. These protozoa may represent an environment that increases their resistance to disinfectants and harsh environmental conditions (Thomas *et al*, 2006; Goy *et al*, 2007). Recently, it was shown that pathogenic subtype I *M. kansasii* strains grew better in *Acanthamoeba castellani* than non-pathogenic subtype III *M. kansasii* strain (Goy *et al*, 2007). Protozoa may, therefore, represent an environment for enhancing the virulence and ability of mycobacteria to spread to vulnerable persons through water (Winiecka-Krusnell and Linder, 2001; Thomas *et al*, 2006; Goy *et al*, 2007).

South African gold miners have an unusually high incidence of *M. kansasii* infection that is attributable to the high prevalence of risk factors for NTM disease, including HIV infection, high incidence of tuberculosis, silicosis, extensive use of aerosolised water for dust control and an unusually high occupational exposure to NTM organisms (Corbett *et*

al, 1999; Corbett *et al*, 2000; Churchyard, 2000). In spite of the identification of these risk factors and a high incidence of *M. kansasii* disease in gold-mining regions of South Africa, the sources of infection for *M. kansasii* disease remain an enigma since *M. kansasii* has not been previously isolated despite previous attempts, precluding any epidemiological link.

3.2 Objective of the Study

The objective of this study was to determine whether mining and/or residential water was the potential source of *M. kansasii* infection for the gold-mining workforce. Attention was focused on water distribution systems since they appeared to be a major source of the mycobacterium in other studies (Engel *et al*, 1980; Steadham, 1980; Chobot *et al*, 1997; Vaerewijck *et al*, 2005). Provision of such information would aid in devising strategies for preventing or reducing transmission of disease caused by *M. kansasii* and other potentially pathogenic mycobacteria to the miners.

3.3 Results

3.3.1 Experimental Design

This was a descriptive study carried out on biofilm and water samples obtained from the Vaal River gold-mining region and the Secunda coal-mining complex. The identity of the *M. kansasii* and other mycobacterial isolates was performed by biochemical tests, PCR and DNA sequencing. Subtyping of *M. kansasii* isolates was carried out by PRA, whilst the determination of an epidemiological link between environmental and clinical isolates was performed by macrorestriction analysis using PFGE. Refer to Chapter 2 for experimental details.

3.3.2 Optimisation of the Isolation Procedure for *M. kansasii* from Water Samples

To select a suitable method for decontaminating samples for the isolation of *M. kansasii*, five commonly-used water decontamination methods were compared. As shown in Table 3.1, 0.005% CPC yielded more mycobacteria (275 CFU/L) than the other decontaminants, followed by 1% NaOH plus 3% SDS, which yielded only 15 CFU/L. The other decontaminants yielded no mycobacteria. In view of these results, CPC was selected as suitable decontaminant for this study. This was in agreement with results from other studies (Neumann *et al*, 1997; Radomski *et al*, 2010).

Table 3.1: Effect of different concentrations of NaOH with 3% SDS and CPC on the isolation of *M. kansasii* from water

Decontaminant	Number of CFU/L plated				
	10^1	10^2	10^3	10^4	10^5
1% NaOH + 3% SDS	NG	NG	NG	NG	15
2% NaOH + 3% SDS	NG	NG	NG	NG	NG
3% NaOH + 3% SDS	NG	NG	NG	NG	NG
4% NaOH + 3% SDS	NG	NG	NG	NG	NG
0.005% CPC	NG	10	25	45	275

NG = No Growth; CFU = Colony Forming Units; CPC = Cetylpyridinium chloride

3.3.3 Isolation of Mycobacteria

A total of 57 (27 water and 30 biofilm) samples from 35 sites in the Vaal River gold-mining region, and 32 (16 water and 16 biofilm) samples from 18 sites in the Secunda coal-mining complex were examined. Culture results showed that 37/57 (64.9%) of the samples from the Vaal River gold-mining region were positive for NTM (Table 3.2). Out of these, 29/37 (78.4%) were isolated from biofilm samples, while 8/37 (21.6%) were isolated from water. For samples from the Secunda coal-mining complex, 17/32 (53.1%) of the samples were positive for NTM (Table 3.2). Sixteen (94.1%) of these NTM were isolated from biofilm samples and only 1 (5.9%) of the mycobacteria was isolated from water samples. Only one underground sample (biofilm) from the Vaal River gold-mining region was positive for NTM, while none was positive from the Secunda coal-mining complex. Counts in biofilm samples ranged from 10 to 233 CFU/swab, while those from water samples ranged from 17 to 197 CFU/L. For both regions, the majority of the NTMs were isolated from biofilm samples obtained from residential and office water distribution systems (Appendix II). Most of the NTM were isolated at 30^{o}C. In spite of measures to control contamination with CPC, fungal overgrowth was a major problem with some water samples. This was particularly evident with surface water samples from the Secunda coal-mining complex.

Table 3.2. Distribution of samples positive for NTM species isolated from water and biofilm samples obtained from the Vaal River gold-mining and Secunda coal-mining complex water distributing systems

Mycobacterial Isolate	Surface water		Underground water		Biofilm samples		All samples	
	No. of Positive Samples	%	No. of Positive Samples	%	No. of Positive Samples	%	No. of Positive Samples	%
Vaal River gold-mining region	8	34.7	0	0.0	29	96.6	37	64.9
Potentially pathogenic NTM	6	26.0	0	0.0	19	63.3	25	43.9
M. kansasii	0	0.0	0	0.0	5	16.7	5	8.8
M. avium	0	0.0	0	0.0	4	13.1	4	7.0
M. fortuitum	4	17.4	0	0.0	3	10.0	7	12.3
M. peregrinum	1	4.3	0	0.0	1	3.3	2	3.5
M. chelonae	0	0.0	0	0.0	3	10.0	3	5.3
M. abscessus	0	0.0	0	0.0	1	3.3	1	1.8
M. parascrofulaceum	1	0.0	0	0.0	1	3.3	1	1.8
M. montefiorense	1	4.3	0	0.0	0	0.0	1	1.8
M. setense	0	0.0	0	0.0	1	1.8	1	1.8
Saprophytic NTM	2	8.7	0	0.0	10	33.3	12	21.0
M. goodii	0	0.0	0	0.0	1	3.3	1	1.8
M. gordonae	2	8.7	0	0.0	9	30.0	11	19.2
Secunda coal-mining complex	1	7.1	0	0.0	16	100.0	17	53.1
Potentially pathogenic NTM	0	0.0	0	0.0	11	68.8	11	34.4
M. avium	0	0.0	0	0.0	2	25.5	2	6.3
M. intracellulare	0	0.0	0	0.0	1	6.3	1	3.1
M. peregrinum	0	0.0	0	0.0	3	18.8	3	9.4
M. chelonae	0	0.0	0	0.0	4	25.0	4	12.5
M. tusciae	0	0.0	0	0.0	1	6.3	1	3.1
Saprophytic NTM	1	7.1	0	0.0	5	31.3	6	18.8
M. gordonae	1	7.1	0	0.0	5	31.3	6	18.8

- Total number of samples from the Vaal River gold-mining region = 57 (biofilms = 30; surface water = 23; underground water = 4). Culture positive, 37/57 (64.9%). Of these positive samples, 29/37 (78.4%) were recovered from biofilms and 8/37 (21.6%) were recovered from water.
- Total number of samples from the Secunda coal-mining complex = 32 (biofilms = 16; surface water = 14; underground water = 2). Culture positive, 17/32 (53.1%). Of these positive samples, 16/17 (94.1%) were recovered from biofilms and 1/17 (5.9%) were recovered from water. NTM, Nontuberculous mycobacteria.

3.3.4 Identification of Mycobacteria

Based on culture, biochemical tests, PCR and DNA sequencing results, several NTM species, including potentially pathogenic and saprophytic species, were isolated from samples obtained from the two regions (Tables 3.2; Appendix II). Potentially pathogenic mycobacteria from the Vaal River gold-mining region included 5 (8.8%) *M. kansasii*, 4 (7.0%) *M. avium*, 7 (12.3%) *M. fortuitum*, 2 (3.5%) *M. peregrinum*, 3 (5.3%) *M. chelonae*, 1 (1.8%) *M. abscessus* and 1 (1.8%) *M. parascrofulaceum*, 1 (1.8%) *M. setense*, 1 (1.8%) and *M. montefiorense*, while saprophytic species included 11 (19.2%) *M. gordonae* and 1 (1.8%) *M. goodii* (Table 3.2). Potentially pathogenic mycobacteria from the Secunda coal-mining complex included 2 (6.3%) *M. avium*, 1 (3.1%) *M. intracellulare*, 3 (9.4%) *M. peregrinum* and 4 (12.5%) *M. chelonae,* 1 (3.1%) *M. tusciae*, whereas saprophytic mycobacteria included 6 (18.8%) *M. gordonae* (Table 3.2). No *M. kansasii* were isolated from the Secunda coal-mining complex. In both regions, potentially pathogenic NTM represented the majority of the isolates, 43.9% and 34.4%, respectively (Table 3.2). Most of the potentially pathogenic NTM were isolated from biofilm samples obtained from showerheads and water taps. Showerhead biofilms were the main sources for *M. kansasii* isolates (Appendix II).

3.3.5 PCR Identification and PRA Analysis of *M. kansasii* Isolates

To identify the five strains of *M. kansasii* isolated from the Vaal River gold-mining region, a PCR-based identification test, using Fw1 and Rv1 primers, was performed (Chapter 2). All five isolates were positive for *M. kansasii*, yielding a 167bp PCR amplified product (Fig 3.1). None of the other mycobacterial species tested (*M. gastri*, *M. tuberculosis*, *M. bovis*, *M. scrofulaceum*, *M. gordonae*, *M. avium*, *M. intracellulare*, *M. xenopi*, *M. fortuitum, M. abscessus, M. peregrinum* and *M. chelonae*) were positive with

this PCR test. The PCR test results were confirmed to be true positives by DNA sequencing of the internal transcribed sequence (ITS) using the ITS1-ITS2 primers.

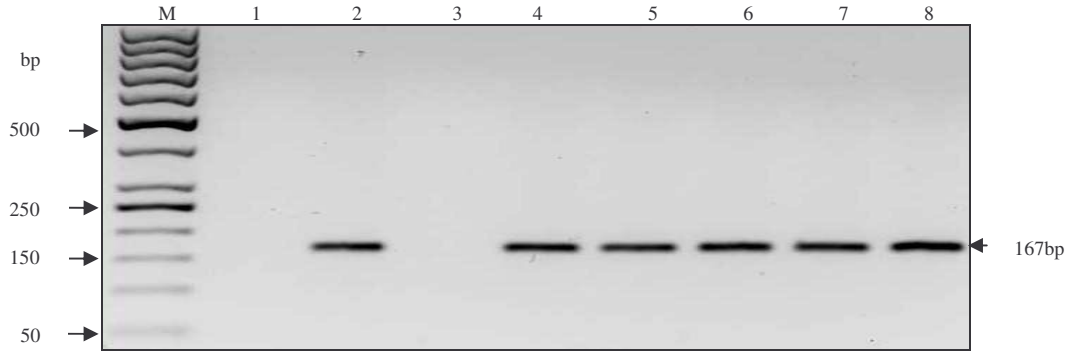


Figure 3.1: PCR detection of environmental isolates of *M. kansasii*. Lane M, 50bp DNA marker; lane 1, No DNA control (water); lanes 2, *M. kansasii* ATCC 12478; lane 3, *M. gastri*; lane 4-8, *M. kansasii* isolates: lane 4, LRRSH-1; lane 5, WSQR; lane 6, KPUGW; lane 7, LRR; and lane 8, NCHSH. bp, base pair.

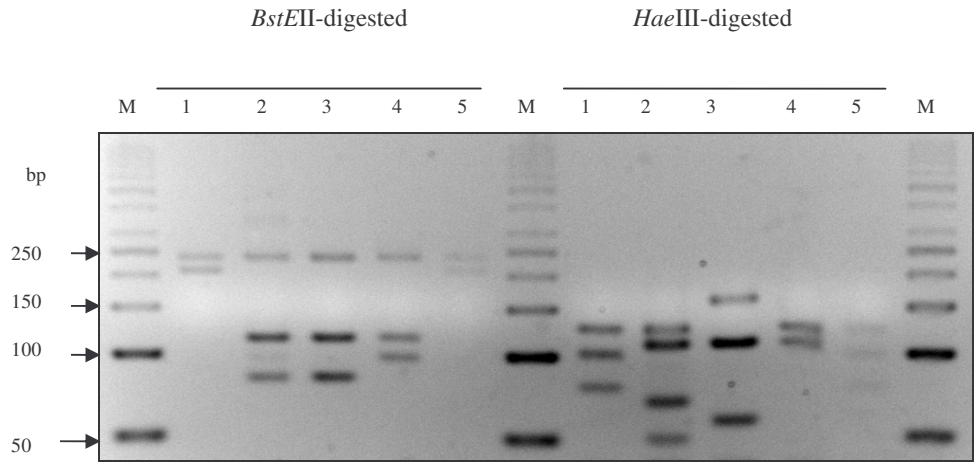


Figure. 3.2: PRA patterns of representative environmental isolates of *M. kansasii*. Lane M, 50bp DNA marker; lane 1, subtype I (LRRSH-1); lane 2, subtype IV (LRR); lane 3, unique isolate 1 (NCHSH); lane 4, subtype II (Clinical isolate); and lane 5, subtype I (WSQR). bp, base pair.

To determine the subtypes of the *M. kansasii* isolates, PRA was performed on the five isolates and three isolates (LRRSH-1, KPUGW and WSQR) were classified into subtype I (*BstE*II, 240/210; *Hae*III, 130/105/80), one isolate (LRR) into subtype IV (*BstE*II, 240/120/85; *Hae*III, 130/115/70/50), and one isolate (NCHSH identified as unique isolate 1) did not match the typical *M. kansasii* PRA pattern (*BstE*II, 240/120/85; *Hae*III, 165/115/60) (Fig 3.2). The isolate with the unique PRA pattern was re-confirmed as belonging to the *M. kansasii* species by DNA sequencing of the 16S-23S rDNA spacer region, the *gyrB* and the *secA1* genes.

3.3.6 Macrorestriction Analysis of Environmental and Clinical Isolates

To determine whether water was the possible source of *M. kansasii* infection for the gold miners, the PFGE macrorestriction profiles of the three subtype I environmental isolates were compared to subtype I *M. kansasii* clinical isolates. The results demonstrated at least 83.5% similarity amongst the isolates, i.e, were categorised as a cluster (Fig 3.3). Particularly striking was the finding that LRRSH-1, an isolate from a mine hostel showerhead, showed similarity of 100% with clinical isolate MK010, and was 91.7% identical to clinical isolates MK128, MK167 and MK221 and 88.0% to MK092. The other two environmental isolates, KPUGW and WSQR, obtained from a mine underground water pipe biofilm and a mine hostel showerhead, respectively, were 100% identical to each other and were 83.5% similar to LRRSH-1 and the clinical isolates (Figure 3.3). These isolates were obtained from three different sites.

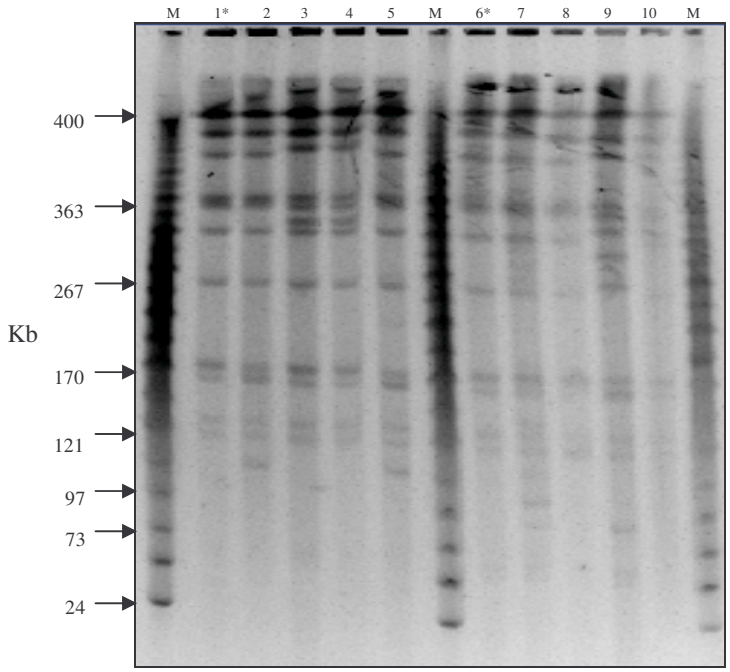


Figure 3.3: Macrorestriction analysis of genomic DNA from clinical and environmental isolates of *M. kansasii* . Lane M, PFG MidRange II marker (NEB); Lane 1, LRRSH-1*; lane 2, MK072, lane 3, KPUGW; lane 4, WSQR; lane 5, MK008; lane 6, MK010*, lane 7, MK078; lane 8, MK015; lane 9, MK027; lane 10, MK165). LRRSH-1, KPUGW and WSQR are environmental isolates, while the rest are clinical isolates. Numbers above the lanes represent isolates and M denotes the molecular weight marker.

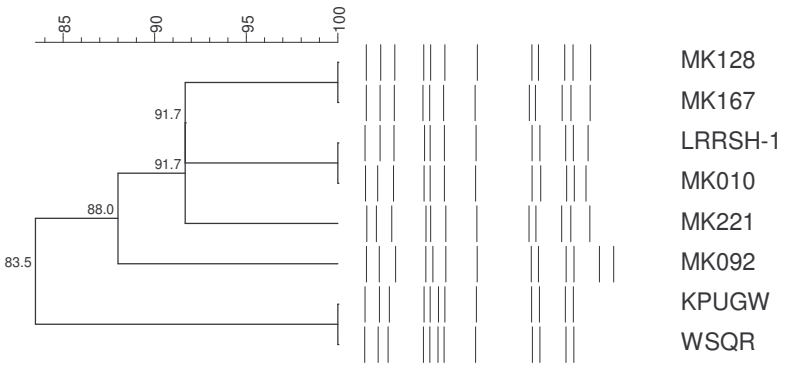


Figure 3.4: Dendrogram and schematic representation of macrorestriction, using PFGE, patterns of environmental (LRRSH-1*, KPUGW and WSQR) and clinical (MK010*, MK092, MK128 and MK167) isolates of *M. kansasii* subtype I. The clinical isolates were selected based on the highest similarity to the environmental isolates

3.4 Discussion

Data presented in this study demonstrate the occurrence of a variety of culturable NTM obtained from water distribution systems in the Vaal River gold-mining region and in the Secunda coal-mining complex. The majority of the mycobacteria were potentially pathogenic species such as *M. kansasii*, *M. avium*, *M. peregrinum*, *M. chelonae*, *M. fortuitum, M. parascrofulaceum* and *M. intracellulare M. montefiorense*, *M. setense* and *M. tusciae*. Saprophytic mycobacteria were also isolated and these included *M. goodii* and *M. gordonae*. Potentially pathogenic mycobacteria other than *M. kansasii* were isolated from biofilms obtained from showerheads and water taps. *M. kansasii* were isolated from biofilms from showerheads and from a mine underground water pipe. Biofilms are significant sources of mycobacteria in water distribution systems (Falkinham *et al*, 2001). A recent study employing a culture-independent molecular survey of the nature of showerhead microbiology in the USA showed that the majority of the bacteria detected were NTM, and that *M. avium* represented the highest load of the mycobacteria (Feazel *et al*, 2009). This was attributable to the enrichment effect showerheads have on bacterial populations and to the waxy content of mycobacteria that imparts resistance to shear forces generated by shower operation. A South African study reported the presence of NTM in 18% of the 78 biofilm samples collected from urban and semi-urban sources (September *et al*, 2004). Nearly all the NTM strains isolated were potential pathogens, which included *M. fortuitum*, *M. abscessus* and *M. septicum*. However, no *M. kansasii* was found. Several other studies have also reported NTM to occur in biofilms of water distribution systems (Falkinham *et al*, 2001; Le Dantec *et al*, 2002; Falkinham *et al*, 2008). Our findings, along with those from other studies, indicate that exposure to water in distribution water systems could pose a health risk, especially to immunocompromised individuals (Falkinham *et al*, 2001; Le Dantec *et al*, 2002; Torvinen *et al*, 2004).

Showerheads, in particular, pose the greatest risk of infection with pathogenic NTM (Falkinham *et al*, 2008; Feazel *et al*, 2009). Although previous epidemiological studies in South Africa have identified predisposing factors such as HIV infection and occupational lung disease as being strongly associated with an increased risk of NTM disease (Corbett *et al*, 1999abc; 2000), it was not possible for us to correlate our data with the incidence and severity of these infections in the South African mining areas as we did not have access to patient data and no scientific record of this information is yet available on mine or other workers in South Africa. This risk of NTM infection in workers and the general public is highlighted in numerous international publications. At present there are no epidemiological data with which to assess the risk for NTM infection.

The five strains of *M. kansasii* were isolated from the Vaal River gold-mining region, and none were isolated from the Secunda coal-mining complex. The strains isolated included three subtype I, one subtype IV and a unique isolate that did not show the typical *M. kansasii* PRA pattern. This unique isolate may probably represent a new *M. kansasii* subtype. It is not clear whether the detection of *M. kansasii* in this region was unusual as systematic studies of other South African sites need to be done to assess the local prevalence. Reports have shown that there is a high prevalence of risk factors for NTM disease in this region (Corbett *et al*, 1999; Churchyard, 2000; Corbett *et al*, 2000), and these risk factors may predispose the mining workforce to *M. kansasii* disease. However, a previous attempt at isolating the organism from water distribution systems in this region failed (G. J Churchyard, personal communication), making it difficult to link the infection to the environment. Only other pathogenic mycobacteria, including *M. scrofulaceum* and *M. avium* complex, were isolated. *M. scrofulaceum* was the most common species isolated, and previously this organism was reported to be the second most important cause

of NTM disease, after *M. kansasii*, in the workforce of this mining region (Corbett *et al*, 1999). Failure to isolate *M. kansasii* from water can occur due to technical issues, and its isolation from water has been reported to be intermittent (Joynson, 1979). Data in our study show that *M. kansasii* is found both in underground mine water and in the domestic water supply of the mine residences. To our knowledge, this is the first study in South African to report the isolation of *M. kansasii* from the environment.

It was initially thought that underground water could have been the possible source of infection since the gold mines use aerosolised water extensively for settling dust (Churchyard *et al*, 2000). However, macrorestriction analysis showed that an *M. kansasii* environmental isolate, from a mine hostel showerhead biofilm, and a clinical isolate were 100% similar to each other. This suggests that potable water may be a possible source of *M. kansasii* infection for the miners in the Vaal River gold-mining region. Aerosols generated from activities such as bathing and showering may put the miners at risk of acquiring infection caused by *M. kansasii*. However, these data do not establish that water is the source of the infection, but implicates it as a possible source of infection. In the absence of an experimental infection model, it is difficult to prove that the miners acquired the infection from water. It may be difficult to reduce the possible exposure of the miners to pathogenic mycobacteria in water, but susceptible individuals should be advised to wear masks while engaging in activities that generate aerosols with water. Other measures to minimise the risk of infection to the miners can include repeated (fortnightly) mechanical cleaning of service water tanks and steaming of the pipeline system, including shower heads, using water heated to 85^{o}C or 90^{o}C for 30min; continuous warming of water to 85^{o}C and its subsequent cooling to 40^{o}C performed once a month; continuous ozone treatment of water; and the installation of ultra-violet (UV) radiation devices in hot water

distribution systems (Chobot *et al*, 1997; Martinková *et al*, 2001). It may also be necessary to slow-down the water flow rate to make these measures more effective (Martinková *et al*, 2001).

3.5 Conclusion

NTM were isolated from 64.9% and 53.1% of the samples obtained from the Vaal River gold-mining region and the Secunda coal-mining complex, respectively. Most of the NTM were isolated from biofilm samples, which included both potentially pathogenic (43.9% from the Vaal River region and 34.4% from the Secunda region) and saprophytic mycobacteria. Of interest was the isolation of *M. kansasii* strains from the Vaal River region. One of the isolates detected had a unique PRA pattern and may probably represent a new *M. kansasii* subtype. Another significant finding was the detection of an *M. kansasii* subtype I isolate that was found to share 100% similarity with a clinical isolate. This study indicates that exposure to water distributing systems containing potentially pathogenic NTM could pose a health risk to people, especially to individuals who are immunocompromised, and that the possible source of *M. kansasii* infection in the mine workforce in the Vaal River gold-mining region is water.

The study had a number of limitations. Some of the negative findings, especially with water samples, may be attributed to the limited number of samples and sites studied. Only one gold-mining region was studied. In addition, the decontamination procedure for the samples, prior to plating on solid media, may have reduced the number of culturable NTM since this has been shown to kill bacteria (Brooks *et al*, 1984). Another limitation of the study was that most NTM are difficult to culture and grow very slowly, making them

vulnerable to overgrowth by other organisms (Brooks *et al*, 1984). A problem observed in this study was with fungal overgrowth, especially with water samples.

Further studies are needed to examine the factors that influence the survival and growth of *M. kansasii* in water distribution systems and the development of an infection model to demonstrate transmission of *M. kansasii* from water to humans.

CHAPTER 4

Genetic Diversity Amongst Clinical and Environmental Isolates of *M. kansasii*

CHAPTER 4

Genetic Diversity Amongst Clinical and Environmental Isolates of *M. kansasii*

4.0 Abstract

The incidence of *M. kansasii* disease in the South African gold-mining workforce is one of the highest in the world (Corbett *et al*, 2000). However, nothing is known about the subtypes or the molecular epidemiology of *M. kansasii* in the country. This study investigated the genetic diversity 191 clinical and environmental isolates obtained from a gold-mining workforce and its environment. *M. kansasii* subtypes were defined by PCR-restriction analysis of the *hsp65* gene. *M. kansasii* subtype I strains comprised 157 (82.2%) of the isolates, while subtypes II and IV comprised 8 (4.2%) and 5 (2.6%) of the isolates, respectively. Two sets of isolates, a group of 16 (8.4%) and another of 5 (2.6%), did not show the typical *M. kansasii* PRA patterns. These isolates were confirmed to belong to the *M. kansasii* species by DNA sequencing of the 16S-23S rDNA spacer region, the *gyrB* and *secA*1 genes, and probably represent new subtypes. Macrorestriction analysis of 114 clinical and 3 environmental subtype I isolates segregated the strains into 12 clusters that showed at least 80% similarity. The strains showed genetic diversity with clustering, but with no association of any cluster with the location of the hospital attended by patients. Taken together, these results showed that *M. kansasii* disease in the gold mining workforce was almost exclusively caused by subtype I strains and that these strains exhibited genetic diversity with no association of clusters with the area of residence of the patients.

4.1 Introduction

The understanding of pathogen distribution and genetic relatedness within species is essential in the determination of the epidemiology of infectious diseases and designing of effective intervention (Singh *et al*, 2006). This can be achieved through the use of molecular typing techniques such as macrorestriction analysis, which utilises infrequently cutting restriction endonucleases to fragment complete bacterial genomes, followed by separation of DNA fragments by PFGE to give reliable DNA fingerprints. Macrorestriction analysis compares the presence or absence of restriction sites, as well as the length of fragments generated (Blanc, 2004; Andrei and Zervos, 2006; Singh *et al*, 2006). Substitutions, insertions, deletions, duplications and rearrangements in the nucleotide sequences change the relative position of the restriction sites and thus change the length of the DNA fragments generated. Differences in the number of DNA fragments generated define strain diversity (Tenover *et al*, 1995).

The epidemiology of *M. kansasii* disease is poorly understood. This is partly attributable to the lack of an effective molecular epidemiological typing system for *M. kansasii*. In addition, the investigation of the reservoir, routes of transmission and pathogenicity of *M. kansasii* has been complicated by the existence of about 7 subtypes of *M. kansasii* (Picardeau *et al*, 1997; Taillard *et al*, 2003; Santin *et al*, 2004*)*. This heterogeneity in the *M. kansasii* species has been demonstrated by the use of PCR restriction analysis (PRA) of the *hsp65* gene (Telenti *et al*, 1993; Picardeau *et al*, 1997; Chimara *et al*, 2008). *M. kansasii* subtype I is the most frequently isolated subtype from clinical sources, while the remaining subtypes are mainly isolated from the environment (Alcaide *et al*, 1997; Zhang *et al*, 2004). However, *M. kansasii* disease has not been demonstrated to be

communicable, and the environment appears to be the source of infection (Engel *et al*, 1980; Steadham, 1980; Chobot *et al*, 1997; Vaerewijck *et al*, 2005).

The epidemiology of *M. kansasii* disease is predominantly urban and has been associated with high density and low income communities (Chobot *et al*, 1997; Bloch *et al*, 1998; Churchyard, 2000; Santin *et al*, 2004). In South African gold miners, there is an unusually high incidence of *M. kansasii* disease which has been estimated to be about 320 per 100 000 (Corbett *et al*, 2000). The structure of the mines and health system in the mining regions make it easy to measure this incidence, but its incidence is not known in the non-mining population of South Africa.

South African gold miners have a high occurrence of risk factors for NTM disease, including HIV infection, a high incidence of tuberculosis, silicosis and considerable occupational exposure to NTM organisms due to the extensive use of aerosolised water for dust control by the gold mines (Churchyard, 2000). These miners are mostly a diverse mobile group of migrant workers from rural South Africa and neighbouring countries. Most of them live in shared rooms in densely populated mine hostels, which may increase the chances of person-to-person transmission of a range of organisms (G. J. Churchyard, personal communication). Despite the high incidence of *M. kansasii* disease amongst South African gold miners, no epidemiological studies have been conducted to determine the genetic diversity of *M. kansasii* isolates from patients.

4.2 Objective of the Study

The objective of this study was to determine the genetic diversity amongst the clinical and environmental isolates of *M. kansasii* obtained from a South African gold-mining region. Determination of the clonal identity of *M. kansasii* strains is important in understanding whether patients are infected with the same or different strains of *M. kansasii* and provides information for understanding and controlling the spread of *M. kansasii* disease.

4.3 Results

4.3.1 Experimental Design

PCR restriction analysis (PRA) of the *hsp65* gene and macrorestriction analysis were used to subtype *M. kansasii* isolates and to determine their clonality, respectively (Refer to Chapter 2 for experimental details). Sixty-seven *M. kansasii* clinical isolates from the town of Orkney and 47 isolates from the town of Carltonville were obtained from patients presenting with confirmed pulmonary *M. kansasii* disease. Both these towns are located in the gold-mining region of the North-West Province. All samples were processed at the West Vaal Hospital TB laboratory in Orkney. No patient information was available due to a lack of access to patient records.

4.3.2 PCR-Restriction Enzyme Analysis

To investigate the distribution of subtypes among *M. kansasii* isolates, 191 single patient isolates were analysed by PRA. Consistent with previous findings (Alcaide *et al*, 1997; Zhang *et al*, 2004), subtype I was found to be the predominant subtype (157, 82.2%), followed by subtype II (8, 4.2%) and subtype IV (5, 2.6%) (Table 4.1). Subtypes III, V, VI and VII were not detected in this study. Unexpectedly, two sets of isolates did not produce any of the typical *M. kansasii* PRA patterns. The two sets comprised 16 (8.4%) and 5 (2.6%) isolates, respectively. The *BstE*II digestion pattern for the first 16 isolates resulted in a pattern identical to that of subtype IV (240, 120 and 85bp), while the *Hae*III digestion gave a unique pattern (165, 115 and 60bp). The PRA pattern of this set of isolates was found to be identical to that of environmental isolate NCHSH (Fig. 3.2, Chapter 3). The *Bst*EII digestion pattern for the remaining 5 isolates was identical to that of subtype I isolates (240 and 210bp), while that of *Hae*III was also unique (130 and 105bp) (Table 4.1; Appendix III). The two sets of isolates were confirmed to belong to the *M. kansasii*

species by DNA sequencing of the *gyrB* and the *secA1* genes, and 16S-23S rDNA spacer region (Appendix IV), and may probably represent new subtypes.

Table 4.1. PRA patterns of the *M. kansasii* DNA after *BstE*II and *Hae*III digestion

M. kansasii subtype	*BstE*II fragment size (bp)	*Hae*III fragment size (bp)	Number of isolates in this study (n, %)
1	240/210	130/105/ 80	157 (82.2)
2	240/130/85	130/105	8 (4.2)
3	240/130/85	130/95/70	-
4	240/120/85	130/115/70/50	5 (2.6)
5	325/120	130/95/75/60	-
6	240/130/85	130/100/75	-
7	240/130/85	130/95/80	-
Unique isolate 1	240/120/85	165/115/60	16 (8.4)
Unique isolate 2	240/210	130/105	5 (2.6)

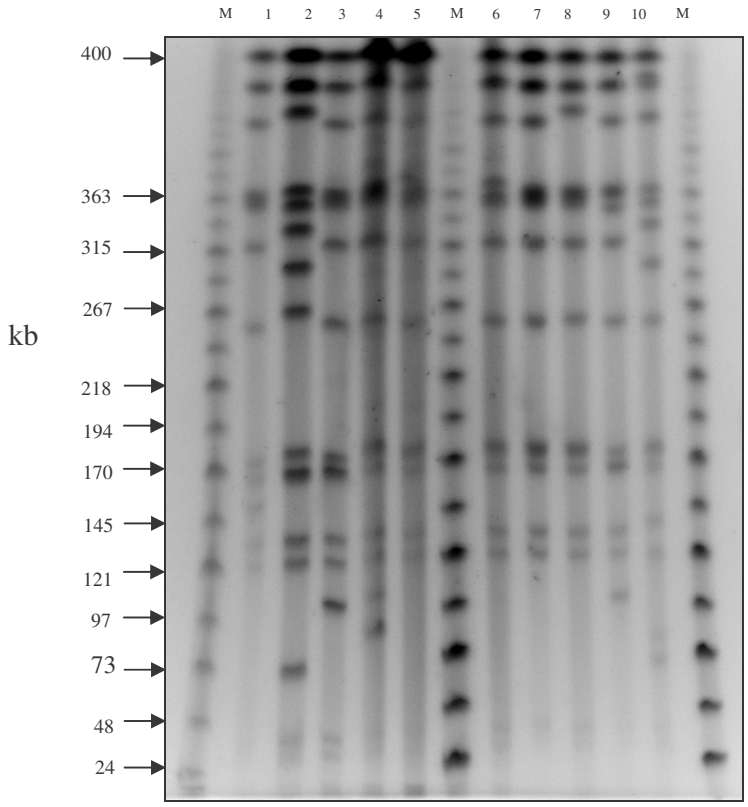


Figure.4.1: Representative gel of macrorestriction analysis, using PFGE, of *M. kansasii* genomic DNA after *Dra*I digestion. Numbers above the lanes represent isolates and M denotes the molecular weight marker, PFG MidRange II (New England BioLabs). kb, kilobase.

4.3.2 Macrorestriction Analysis

Previous studies have indicated that *M. kansasii* subtype I strains are predominantly clonal in nature (Alcaide *et al*, 1997; Picardeau *et al*, 1997; Zhang *et al*, 2004). To investigate whether this was the case with the subtype I strains isolated in our study, macrorestriction analysis was performed on the strains with *Dra*I, which generated fragments ranging in size from about 50 to 400kb (Figure 4.1). An attempt to use *Xba*I was made but the restriction fragments generated were too complex to analyse (refer to Appendix IV). *Dra*I produced about 10 to 17 fragments, which were easily analysable. However, out of the 157 subtype I strains, only 114 were analysable due to problems associated with DNA degradation and fungal contamination. The three subtype I environmental isolates, LRRSH-1, KPUGW and WSQR, were also included in the analysis. In general, the PFGE analysis demonstrated diversity amongst the isolates. When electrophoretic profiles were compared using GelCompar software, 12 clusters, A-L, of at least two isolates whose PFGE patterns were similar by at least 80%, could be observed (Figure 4.3). This similarity index (SI) was determined by comparing three possible SI cut-offs, 75%, 80% and 90%. The SI cut-off that provided the best discrimination, after visual inspection of the dendrogram, was 80% as it correctly assigned the isolates to their respective clusters. Acceptability of these clusters was assessed by visual inspection of gels to check for similarities or identities of the patterns. It was confirmed that macrorestriction patterns of the isolates within each cluster differed by a maximum of six bands, which is suggested to correspond to two mutations and is considered to represent a significant level of genetic relatedness (Tenover *et al*, 1995). Isolates with seven or more distinct bands were designated “unrelated”. Cluster B contained the largest number of isolates (42) which shared 82.2 to 100% similarity, followed by clusters A (19) and F (15), each with strains sharing 81.0 to 100% and 80.4 to 100% similarities, respectively; clusters E and H were

represented by 7 isolates each, showing similarities of more than 81.3%; clusters C and K each represented by 5 isolates showing a similarity of at least 80.7%; clusters D and L contained 2 isolates, each exhibiting at least 87.0% and 88% similarity, respectively; cluster G was represented by 4 isolates with at least 84.6% similarity; and clusters I and J with 3 isolates each, exhibited at least 82.1% similarity. Only two isolates, including the *M. kansasii* ATCC 12478 reference strain, remained outside these clusters. The most interesting cluster was F, which comprised 14 clinical and 3 environmental isolates. As stated in Chapter 3, one of these isolates, LRRSH-1 showed 100% identity with clinical isolate MK010 (Figure 4.2). The remaining two isolates, KPUGW and WSQR, were also 100% identical to each other but were only 86.1% identical to LRRSH-1 and a number of selected clinical isolates. There was no evidence of clustering or association between PFGE pattern and the location of the hospital attended by patients.

Figure 4.2. Dendrogram showing the genetic relationships amongst the macrorestriction (using PFGE) profiles of *M. kansasii* isolates analysed in this study. An 80% similarity index (SI) (green vertical line) was chosen as it provided best discrimination of the isolates compared to the other two SI cut-offs tested, 75% and 90%.

4.4 Discussion

It has been estimated that the incidence of *M. kansasii* disease in the South African gold-mining workforce is about 320 per 100 000 employees (Corbett *et al*, 2000). However, this figure could be an underestimation of the actual number of cases considering this estimate was made about 10 years ago when the HIV positivity rates may have been lower than those seen currently. A number of predisposing risk factors, including HIV and silicosis, are considered to contribute to this high incidence of disease (Corbett *et al*, 1999; Corbett *et al*, 2000). However, it was not within the scope of this study to determine whether or not these risk factors influenced the clustering patterns obtained.

Molecular epidemiology of human bacterial pathogens provides valuable information for understanding the reservoir, pathogenicity and control of these bacteria (Singh *et al*, 2006). In this study the genetic diversity amongst *M. kansasii* strains isolated from gold miners and their environment was determined using PRA and PFGE analysis. The majority (82.2%) of the patients were infected with subtype I, the most prevalent subspecies from human sources worldwide (Alcaide *et al*, 1997; Gaafar *et al*, 2003; Taillard *et al*, 2003; Santin *et al*, 2004; Zhang *et al*, 2004). The predominance of this subtype may present an association with virulence, conferring upon it enhanced capacity for colonisation and pathogenic activity for humans (Alcaide *et al*, 1997; Taillard *et al*, 2003; Goy *et al*, 2007). In fact a number of studies have shown that this subtype is the predominant subspecies regardless of the HIV status of patients (Chimara *et al*, 2004; Santin *et al*, 2004). Other subtypes detected included subtypes II (4.2%), IV (2.6%) and two sets of isolates with unique PRA restriction patterns not matching any of the typical *M. kansasii* restriction patterns. One set of these isolates matched the PRA restriction pattern of an *M. kansasii* environmental isolate (Chapter 3). This set of isolates was

confirmed to be *M. kansasii* by DNA sequencing of the *gyrB* and the *secA1* genes, and 16S-23S rDNA spacer region. It was also of interest to ascertain whether these new isolates could be detected by the current detection system in our TB laboratory. Interestingly, the AccuProbe assay used in the laboratory was able to identify all of them as being *M. kansasii* species. The two sets of isolates may probably represent new subtypes of *M. kansasii*. The detection of these two sets of isolates in this study may reflect the existence of a unique ecological niche for the bacterium that influences its evolution resulting in the observed change in the PRA restriction pattern (Taillard *et al*, 2003).

Macrorestriction analysis of the subtype I isolates generally demonstrated genetic diversity amongst the isolates, and 12 clusters were observed. Several previous studies have demonstrated a tight clonal structure amongst subtype I strains. One of the first studies by Picardeau and co-workers reported the use of large restriction fragment-PFGE analysis to differentiate 24 subtype I strains which generated 4 patterns with *Dra*I restriction enzyme (Picardeau *et al*, 1997). They found that the strains were clonal in nature when single-enzyme Amplified-Fragment Length Polymorphism (AFLP) analysis was used with *Pst*I enzyme. Only a few strains generated patterns different from those shared by the majority of the isolates. Similar observations were made in a Spanish study which utilised PFGE with *Dra*I. Limited genetic polymorphism was observed amongst the 111 strains tested, which were segregated into 5 clusters with minor differences (Alcaide *et al*, 1997). An American study found similar results with the use of PFGE and restriction enzymes *Dra*I and *Ase*I. It demonstrated marked clonality amongst the isolates studied, with only minor differences (Zhang *et al*, 2004). When compared to isolates from France and Japan, the *Dra*I and *Ase*I pulsotypes showed 44% and 42% similarity, respectively. A more recent

study from Taiwan, using PFGE and *Ase*I, also corroborated the above findings (Wu *et al*, 2009). The 22 subtype I isolates were also found to be clonal in nature. In an attempt to address the tight clonal structure observed in subtype I strains, Gaafar and co-workers evaluated the usefulness of a single-enzyme AFLP method for further differentiating the strains (Gaafar *et al*, 2003). The method was able to segregate the strains into 12 distinct clusters, demonstrating its potential use in the typing of the *M. kansasii* strains.

The underlying reasons for the observed diversity of the strains in our study are unclear but may be caused by the distinct susceptible population at the mines. The high prevalence of risk factors such HIV infection and silicosis, which are not very common in the developed world, could influence the adaption of the strains in the patients, resulting in the observed pattern. In addition the observed diversity could be due to a combined result of recombinational events and unique ecological factors in the South African gold-mining region studied that dictate these changes (Coenye and LiPuma, 2003; Feil *et al*, 2001; Feil and Spratt, 2001).

It is not known whether *M. kansasii* disease is communicable or whether it is acquired from the environment, but evidence does tend to suggest environmental reservoirs as the sources of infection. Nonetheless, two cases of *M. kansasii* infection in the same household have been reported, and this was suspected, but not proven, to be due to intra-familial infection (Penny *et al*, 1982). Another study documented a case involving a mother and a son, both of which were infected with the same strain of *M. kansasii* (Leal-Arranz *et al*, 2005). It was not possible to establish whether the infection was transmitted from one person to another or whether it was acquired from a common source (Leal-Arranz *et al*, 2005). In our study no association of the PFGE pattern and the location of

hospital attended was noted in the two patient populations studied. The diversity of the clinical isolates also supports an environmental origin such as water.

One of the significant findings in this study was the observation that one of the environmental isolates, obtained from a mine hostel showerhead biofilm, showed 100% identity with a clinical isolate (Chapter 3). The clinical isolate was obtained from a patient attending a mine hospital in Cartonville, whilst the environmental isolate was obtained from a mine hostel in Orkney. This may be explained by the fact that there is a high degree of mobility between the gold-mine workforces in the two regions, resulting in possible infection (G. J. Churchyard, personal communication). Though the PFGE pattern for some isolates were 100% identical, it was impossible to assess retrospectively whether transmission had occurred amongst the patients or if infection was acquired from the environment.

4.5 Conclusion

This is the first study undertaken in South Africa to employ molecular tools to subtype strains of *M. kansasii*. The study demonstrated that subtype I is the predominant *M. kansasii* strain causing infection in the gold miners studied and corroborates findings from other parts of the world. Two new restriction patterns were detected using PRA, and may probably reflect the identification of new subtypes of *M. kansasii*. In contrast to the close relationships amongst the Japanese, European, American, and Taiwanese isolates, South African *M. kansasii* subtype I isolates generally exhibited a degree of diversity. No association between the PFGE pattern and hospital location could be observed amongst the patient isolates that clustered together.

One major limitation of this study was the lack of epidemiological data. Unfortunately, these data were not available due to logistical problems and lack of access to patient records. Further work is warranted to determine the distribution of *M. kansasii* strains isolated from other parts of South Africa. This will give an accurate picture of the *M. kansasii strains* circulating in the country.

CHAPTER 5

Genetic Differences Between Clinical and Environmental Isolates of *M. kansasii*

CHAPTER 5

Genetic Differences Between Clinical and Environmental Isolates of *M. kansasii*

5.0 Abstract

M. kansasii species comprises about 7 genetically very similar subtypes, and subtype I is considered to be the most pathogenic of all of the subtypes. However, little is known about the genetic basis for its virulence. To address this, Hybridisation-Monitored Genome Differential Analysis (HMDA) and bioinformatic analysis were used to identify genetic differences between *M. kansasii* subtype I clinical isolate and *M. kansasii* subtypes III, V and VI environmental isolates. Following solid-phase hybridisation, 150 plasmid clones were sequenced to define unique or divergent genomic sequences in subtype I. Putative DNA sequences were analysed by dot blot hybridisation, which confirmed the uniqueness of the isolated fragments. Comparative DNA analysis identified 45 open reading frames (ORFs) predicted to encode putative secreted or membrane-associated proteins. These included six genes encoding putative virulence associated factors (MCE family protein, two polyketide synthases [Pk8 and Pk12], acyltransferase [PaPA5] and an O-methyltransferase), two members of the transcription regulators for drugs and xenobiotics, three members of multidrug efflux systems, those associated with lipid and carbohydrate metabolism and transport, and a number of hypothetical proteins of unknown function. These results indicate that *M. kansasii* subtype I can be distinguished from other subtypes by a number of divergent ORFs, some of which encode virulence-associated factors.

5.1 Introduction

The enormous amount of data generated from whole genome sequencing projects and the availability of comparative genomic tools for analysis of the generated data enable the characterisation of genetic differences in microorganisms. Biological properties such as gene function, virulence traits, immunity and evolution can be inferred from analysis of the full genome sequences (Clark, 1999; Brosch *et al*, 2001; Gordon *et al*, 2002; Behr, 2008). In the context of pathogenic organisms, the results from such studies can help in developing new methods for diagnosis and identification of therapeutic targets (Fitzgerald and Musser, 2001; Randhawa and Bishai, 2002). However, whole genome sequencing of multiple bacterial strains or species is very cumbersome, expensive and, in most cases, impractical for most laboratories (Shanks *et al*, 2006a).

During the past two decades, alternative genomic methods to whole genome sequencing methods have been developed to identify and isolate unique sequences present in one genome but absent in another. Most of these methods are based on subtractive hybridisation, in which target sequences in one population (tester) are enriched by hybridising with an excess amount in another population (driver), resulting in the removal of common sequences between the two populations (Wieland *et al*, 1990; Graham and Clark-Curtiss, 1999; Shanks *et al*, 2006a; Lamar and Palmer, 1984; Lisitsyn *et al*, 1993). The selected tester DNA molecules may be enriched by PCR amplification after each round of subtraction (Shanks *et al*, 2006a) or by consecutive subtraction with PCR amplification only applied after the final subtraction round (Yueqing *et al*, 2006). This approach has not only identified genetic differences among different strains or species but has also defined genes that are associated with virulence of an organism (Tinsely and

Nassif *et al*, 1996; Graham and Clark-Curtiss, 1999; Hou *et al*, 2002; Dwyer *et al*, 2004; Shanks *et al*, 2006a).

Amongst the 7 subtypes of the *M. kansasii*, subtype I is the most frequently isolated subtype from human infection, and is generally regarded as the most pathogenic of the subtypes (Santin *et al*, 2004; Zhang *et al*, 2004; Goy *et al*, 2007). However, nothing is known about the genetic basis for its virulence. A better understanding of the genetic variations amongst the *M. kansasii* strains is needed and will be important in studying the evolution and diversity of pathogenicity. In addition, this will allow the identification of markers for use in the development of accurate diagnostic tools and therapeutic targets.

5.2 Objective of the Study

The objective of this study was to identify genetic differences between an *M. kansasii* subtype I clinical isolate and *M. kansasii* (subtypes II, IV and VI) environmental isolates.

5.3 Results

5.3.1 Experimental Design

HMDA was utilised to directly identify and clone genomic differences between *M. kansasii* subtype I (tester) and *M. kansasii* subtypes III, V and VI (drivers) with a view to identifying divergent DNA segments in subtype I. Experimental details for HMDA are described in Chapter 2 under Section 2.6. Multiple rounds of hybridisation were used to enrich *M. kansasii* subtype I-specific DNA fragments and the hybridisation monitoring process was performed by targeting the 16S rDNA, a gene that is highly conserved amongst bacterial species (Rogall *et al*, 1990; Harmsen *et al*, 2002). The identification of unique subtype I genome fractions in the enriched DNA was performed by dot blotting, DNA sequencing and analysed by the BLASTX programme at the National Center for Biotechnology Information (http://ncbi.mlm.nlm.gov/BLAST). The DNA sequences were then organised into functional gene categories using the Protein Clusters Database (ProtClustDB) (http://www.ncbi.nlm.nih/gov/sites/entrez?db=proteinclusters). BLASTX sequence matches with *E* values of $\leq 1 \times 10^{-3}$ and sequence identities of $\geq 60\%$ were regarded to be similar protein sequences (Pearl *et al*, 2000; Ribeiro-Guimaraes and Pessolani, 2007; Punta and Ofran, 2008). Figure 5.1 below illustrates the principle behind the HMDA approach used.

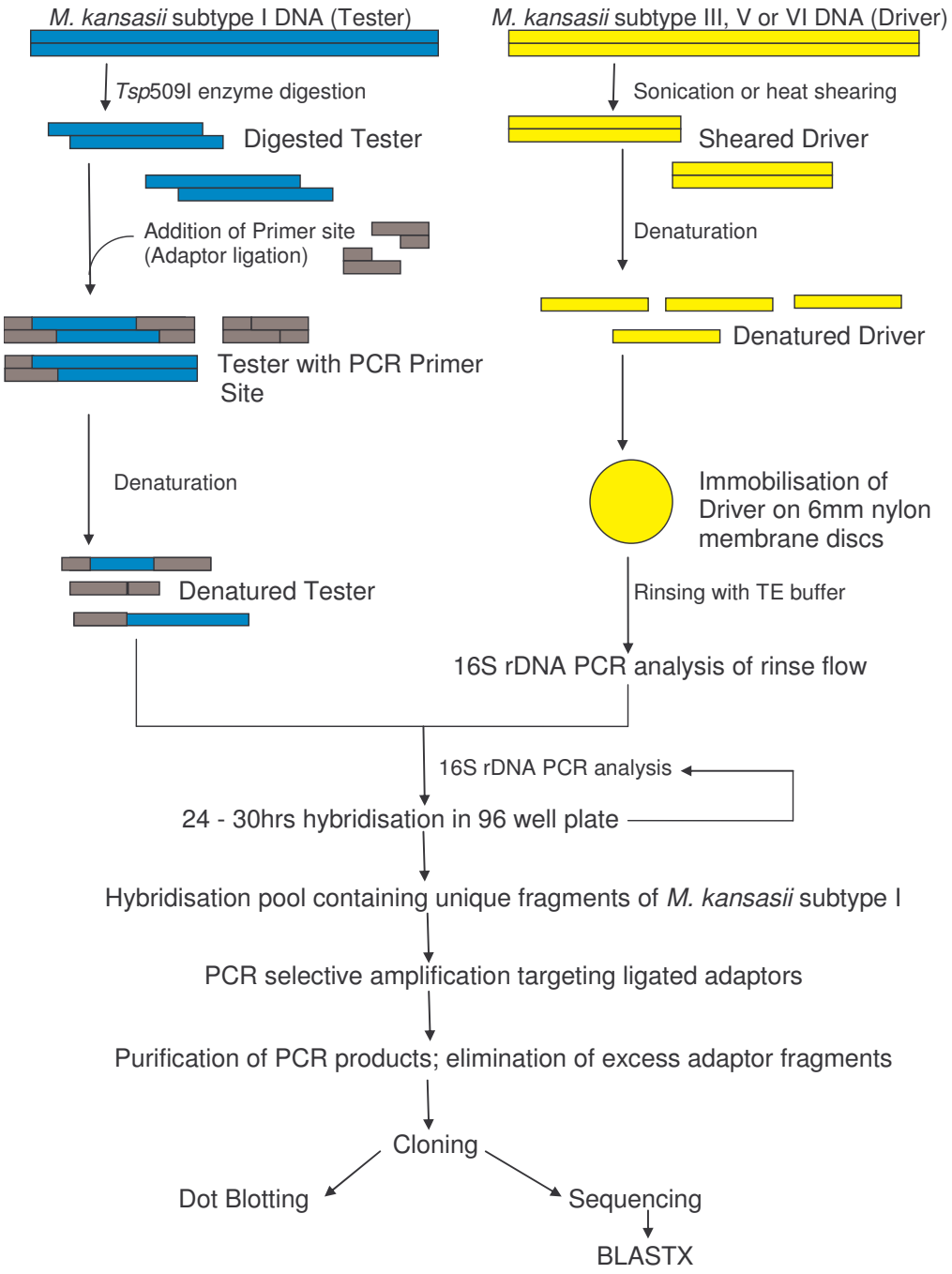


Figure 5.1: Schematic representation of the HMDA strategy used to identify *M. kansasii* subtype I-specific DNA sequences that are absent or divergent in *M. kansasii* subtypes III, V, VI (Adapted from Yueqing *et al*, 2006).

5.3.2 Preparation of Tester and Driver DNA

Tester and driver DNA were prepared by enzymatic digestion and heat fragmentation, respectively. Tester DNA, from an *M. kansasii* subtype I clinical isolate, was prepared by digestion with *Tsp*509I (New England BioLabs, Ipswich, MA) and ligation with a P1 primer-specific PCR target adaptor molecule. Successful preparation of the Tester DNA was confirmed by the presence of PCR fragments ranging from 250 to 2000bp (Figure 5.2).

To prepare driver DNA, environmental *M. kansasii* subtypes III, V and VI were selected and fragmented at 95^{o}C using a time series of 20 to 45min (Figure 5.3). Fragments from 150 to 1000bp (from the 30min fragmented sample) were then selected and immobilized on 6mm diameter Hybond N^{+} Nylon membranes (Amersham Biosciences, Buckinghamshire, UK) for use in the hybridisation process.

5.3.3 Monitoring of the Subtraction Process and Hybridisation

To monitor the efficiency of the subtraction and hybridisation process, PCR analysis of the 16S rDNA in the tester sample was performed. After the fifth round of hybridisation, no 16S rDNA was detectable (Figure 5.4A). This suggested that most of the homologous sequences between the tester and driver DNA populations had been removed and that the remaining fragments were mainly unique to the tester sample.

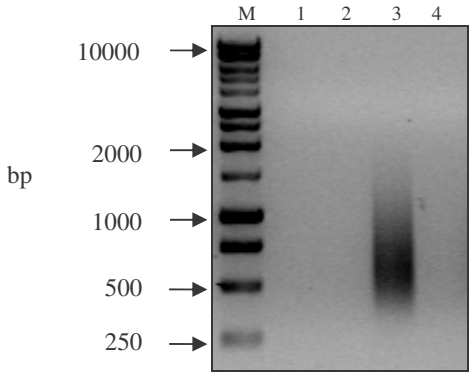


Figure 5.2: Verification for the presence of successful preparation of tester DNA. Lane M, 1 kb marker (Promega); Lane 1, Negative control (water); Lane 2, Negative control (adaptor molecule only); Lane 3, adaptor-tester DNA, Lane 4, Negative control (*Tsp*509I-digested Tester DNA)

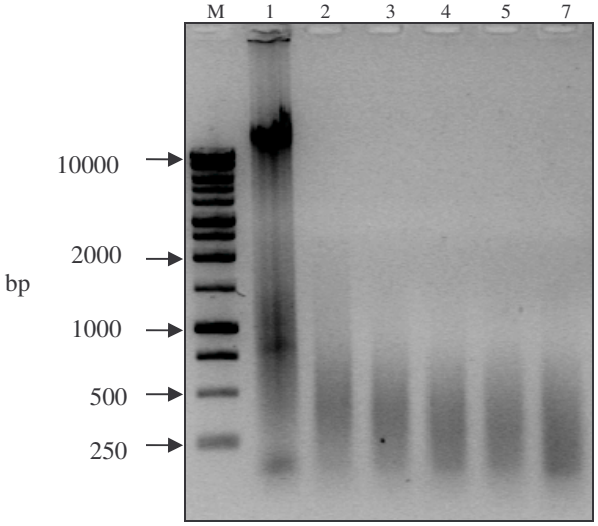


Figure 5.3: Heat (95^{o}C) fragmentation of Driver DNA. Lane M, 1kb DNA marker (Promega); Lane 1, unheated genomic driver DNA; Lane 2-6, heated genomic DNA samples: lane 2, 20min; lane 3, 25min; lane 4, 30min; lane 5, 35min; lane 6, 40min. bp, base pair.

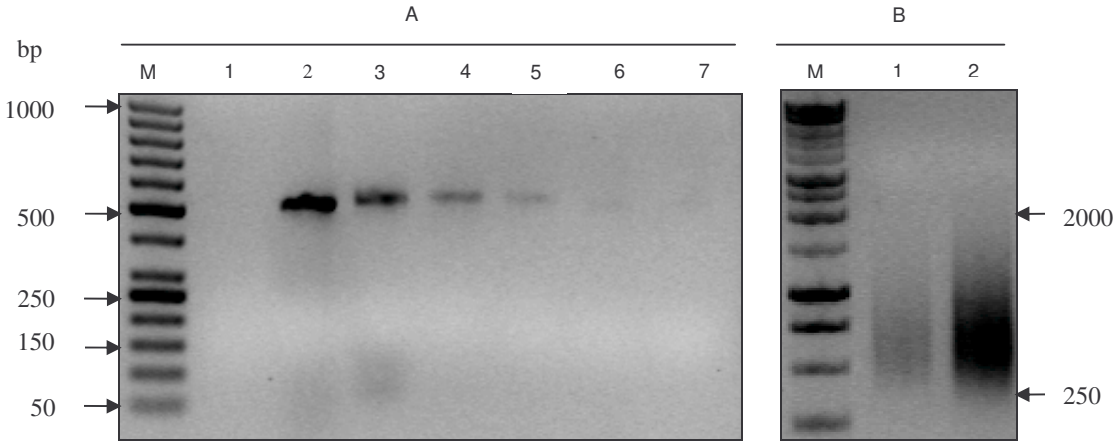


Figure 5.4: Monitoring of the hybridisation process and isolation of *M. kansasii* subtype I-specific DNA fragments. Panel A: Monitoring of the hybridisation process using 16S rDNA. Lane M, 50bp DNA marker (Fermentas); lane 1 = no DNA control; lane 2, positive control (unsubtracted tester DNA), lanes 3 to 7, subtracted tester DNA after 4, 6, 8, 24 and 28 hrs of hybridisation, respectively. Panel B: Isolation of tester DNA not present in the Driver sample through subtractive hybridisation. Lane M, 1 kb DNA marker (Promega); lane 1, final Tester DNA PCR product; lane 2, unsubtracted Tester DNA for comparison. bp, base pair.

Genomic subtraction resulted in the enrichment of *M. kansasii*-specific sequences (Figure 5.4, panel B). Agarose gel electrophoresis of products in the final round of PCR amplification revealed fragments ranging from 300 to 2000bp in the subtracted DNA fractions.

5.3.4 Screening of Cloned DNA Fragments

PCR products from the final round of the hybridisation process were cloned and 135 randomly selected clones were screened for the presence of tester DNA using PCR, restriction enzyme analysis and dot blotting.

To detect the presence of the cloned tester fragments, PCR and enzymatic analysis were performed on DNA extracted from a number of selected clones using the P1 primer and

EcoRI enzyme, respectively. Both screening tests revealed the presence of the tester fragments, ranging from 500 to 1500bp, in the selected clones (Figures 5.5 and 5.6).

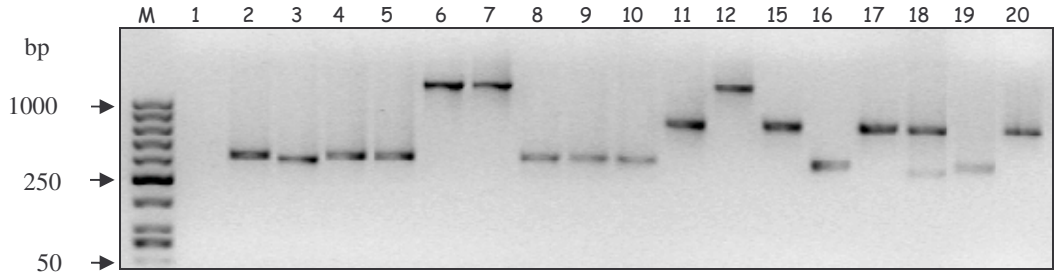


Figure 5.5: PCR-screening of a number of randomly selected clones using the P1 primer. Lane M, 50bp marker (Fermentas); Lane 1, no DNA control; lanes 2 to 20, amplification products of randomly-selected tester clones. bp, base pair.

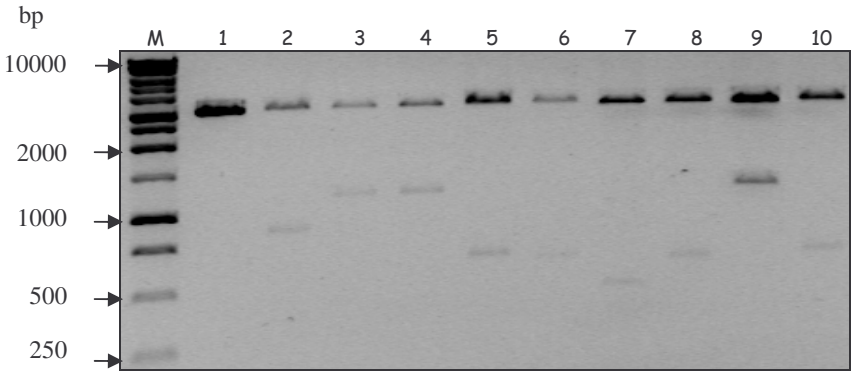


Figure 5.6: Enzymatic screening of a number of randomly selected clones using *EcoRI*. Lane M, 1 kb marker (Promega); Lane 1, uncut pGEM T-Easy vector; lanes 2 to 10, restriction products of randomly-selected tester clones. bp, base pair.

To determine the specificity of the isolated tester DNA fractions, dot blotting was used. Using a subtracted tester and a driver DNA probe, dot blotting confirmed that most of the *M. kansasii* subtype I-specific DNA fragments were unique to the subtype I (Figure 5.7).

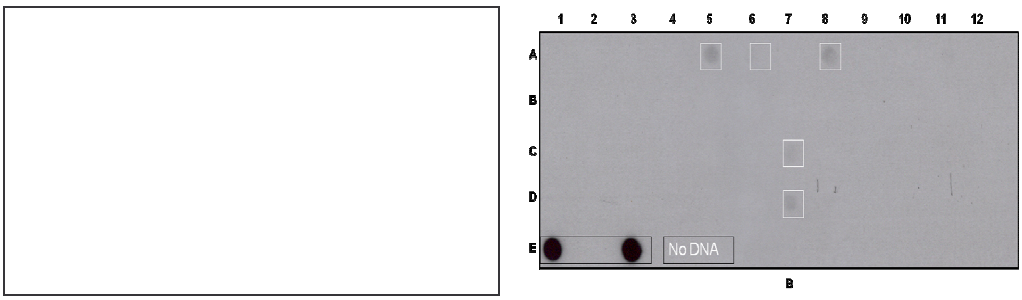


Figure 5.7: Dot blot hybridisation analysis of *M. kansasii*-specific DNA fragments. Positive controls included driver (E1), subtracted and unsubtracted tester (E2 and E3, respectively) genomic DNA. Panels A and B show membranes containing subtracted *M. kansasii* subtype I genomic DNA probed with tester DIG probe and driver DIG probe, respectively.

5.3.5 Analysis of DNA Sequences

A total of 135 clones were analysed for divergent sequences in *M. kansasii* subtype I. Nineteen DNA sequences (14.1%) had no significant matches to the draft genome sequence of the *M. kansasii* ATCC 12478 (subtype I) reference strain by BLASTX analysis (Figure 5.8). The remaining 116 sequences had significant similarity to the sequences in the NR protein database, and a total of 45 open reading frames (ORFs) were detected. Forty-four (32.6%) of these ORFs were detected in *M. kansasii* ATCC 12478 genome and one (0.7%) was detected in *M. gilvum* PYC-GCK genome. Most fragments frequently showed similarity to the same protein, suggesting that the same gene was represented several times within the HMDA library (Table 5.1). All the ORFs detected were assigned to one of the eighteen functional categories described in the Protein Clusters Database (ProtClustDB) (http://www.ncbi.nlm.nih.gov/sites/entrez?db=proteinclusters) (Figure 5.8). The category most frequently assigned was lipid metabolism (10.4%), followed by cell wall and membrane (8.1%), unknown function (7.4%), carbohydrate metabolism and transport (5.2%), secondary metabolism and transport (5.2%), energy metabolism (3.7%), cell secretion (2,2%), amino acid metabolism and transport (2.2%), coenzyme metabolism (1.5%), signal transduction mechanisms (1.5%), nucleotide

metabolism and transport (0.7%), inorganic ion transport (0.7%), molecular chaperones (0.7%), and DNA replication, recombination and repair (0.7%) (Figure 5.8).

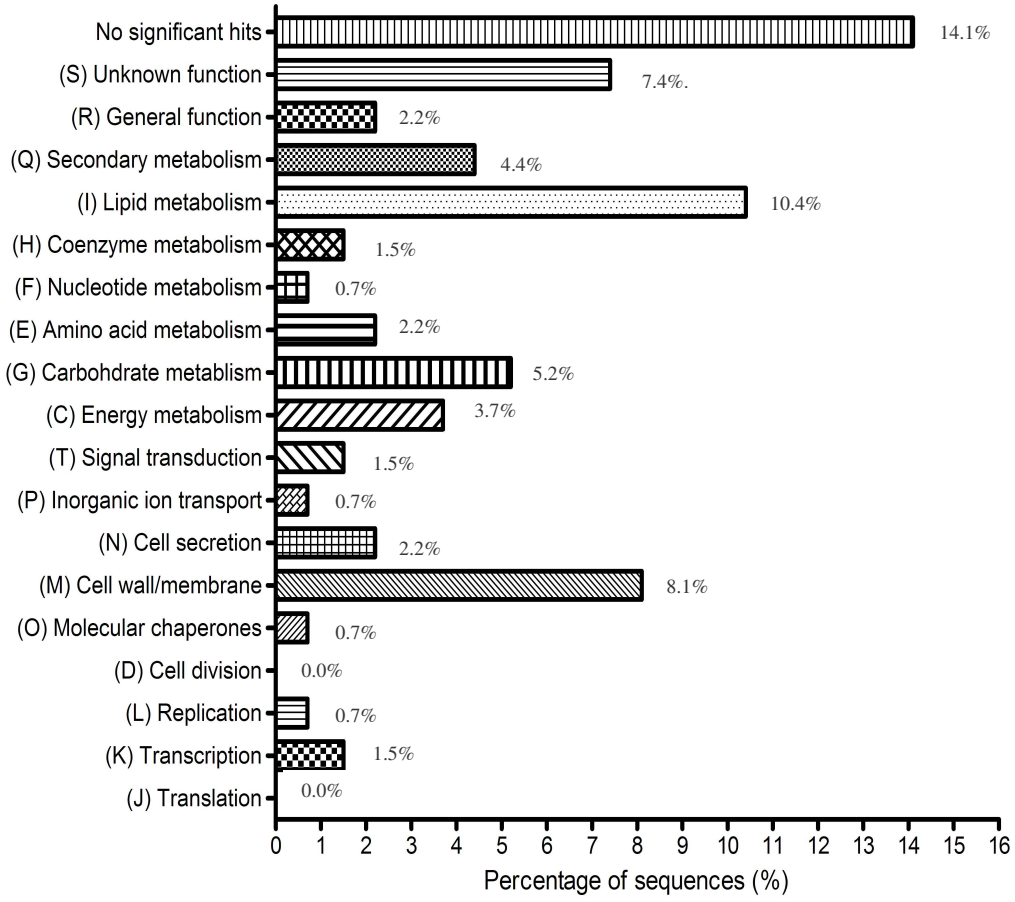


Figure 5.8: Functional group assignments of HMDA-enriched *M. kansasii* subtype I DNA sequences using the Cluster of Orthologous Groups of proteins (COG) classification based on the top BLASTX hits. Sequence matches with *E*-values of $\leq 10^{-3}$ and sequence identities of $\geq 60\%$ were considered to be similar protein sequences. Functional groups are listed on the *y*-axis, while the percentage of the sequences for each group is shown on the *x*-axis (total number of sequences = 135).

Table 5.1: ORFs identified as being divergent in *M. kansasii* subtype I generated from DNA sequences obtained by HMDA

Number of Clones	Top BLASTX Match with *M. kansasii* ATCC 12478	ORF	*E*-value	Proposed Functional Category
29	Conserved hypothetical protein	MkanA1_09104	$8e^{-75}$	Putative lauroyl/myristoyl acyltransferase
14	O-methyltransferase	MkanA1_02222	$2e^{-21}$	Mycolic acid oxygenation; biosynthesis of proteins interfering with DNA-binding antibiotics
1	FeS oxidoreductase	MkanA1_09109	$2e^{-24}$	General catalytic function; arylsulphatase regulator
1	Ydjk	MkanA1_22260	$5e^{-76}$	Permease: facilitates movement of substances across cytoplasmic membrane
1	Endo-1,4-β-glucanase	MkanA1_19721	$1e^{-109}$	Cellulose breakdown to glucose
1	Glycoside hydrolase family protein	MkanA1_07384	$4e^{-57}$	Carbohydrate metabolism
1	Methylmalonyl-CoA mutase α subunit	MkanA1_19676	$2e^{-74}$	Cabalamin-dependent enzyme – propionate acid formation
1	Isopentyl pyrophosphate isomerase	MkanA1_19761	$4e^{-62}$	Catalysis of 3-isopentyl pyrophosphate to dimethylally pyrophosphate (metabolism)
1	MCE family protein	MkanA1_27376	$1e^{-96}$	Host cell invasion; virulence factor for mammalian cell entry (mce)
1	Transmembrane transport protein, MmpL13_1	MkanA1_19751	$3e^{-67}$	Predicted drug exporter of the RND family
3	Putative transmembrane ATP-binding protein	MkanA1_02417	$1e^{-16}$	ABC-type multi-drug transporter
1	TetR family transcription regulator	MkanA1_07199	$1e^{-40}$	Transcription regulation; control of level of antibiotic & detergents
12	Predicted integral membrane protein	MkanA1_28696	$1e^{-26}$	Unknown
8	Predicted integral membrane protein	MkanA1_05680	$5e^{-73}$	Unknown
1	Hypothetical protein	MkanA1_23416	$2e^{-15}$	Unknown
1	Hypothetical protein	MkanA1_24335	$2e^{-15}$	Unknown
3	Hypothetical protein	MkanA1_12371	$7e^{-16}$	Molecular chaperon
1	PE family protein, PE19_1	MkanA1_24070	$6e^{-76}$	Pro-Glu-rich, uncertain function
3	PE-PGRS family protein, PE-PGRS	MkanA1_27316	$1e^{-73}$	Glycine-rich, uncertain function
4	PE-PGRS family protein, PE-PGRS1	MkanA1_29646	$3e^{-26}$	Glycine-rich, uncertain function
1	PE-PGRS family protein, PE-PGRS2	MkanA1_29416	$5e^{-75}$	Glycine-rich, uncertain function
1	PPE family protein	MkanA1_24065	$2e^{-108}$	Pro-Pro-Glu, uncertain function
1	Putative lipoprotein	MkanA1_27726	$1e^{-48}$	Unknown
1	Polyketide synthase, pks8	MkanA1_12468	$3e^{-106}$	Synthesis of mycocerosic acid (fatty acid); Secondary metabolite synthesis, transport & catabolism; virulence-associated
1	Polyketide synthase, pks12	MkanA1_14385	$1e^{-53}$	Synthesis of mycocerosic acid (fatty acid); Secondary metabolite synthesis, transport & catabolism; virulence-associated

continued on next page

Table 5.1: continued from previous page

Number of Clones	Top BLASTX Match with *M. kansasii* ATCC 12478	ORF	*E*-value	Proposed Functional Category
1	Acyltransferase, PaPA5	MkanA1_09847	$4e^{-100}$	Biosynthesis & transport of phthiocerol dimycerosate & phenolic glycolipids; virulence-associated
1	Enoyl-CoA-hydratase	MkanA1_03967	$1e^{-93}$	Lipid transport & metabolism
3	Acetylglutamate kinase	MkanA1_27791	$7e^{-26}$	Amino acid metabolism & transport
2	DNA gyrase subunit A	MkanA1_22465	$2e^{-35}$	DNA replication, recombination and repair
1	Zinc-type alcohol dehydrogenase	MkanA1_02387	$6e^{-26}$	NAD-dependent reversible oxidation of ethanol
1	Hypothetical transmembrane protein	MkanA1_04077	$1e^{-31}$	Chloride channel protein, EriC
2	Short-chain dehydrogenase	MkanA1_21648	$9e^{-99}$	Secondary metabolism: biosynthesis, transport & catabolism
1	3-ketoacyl-(acyl-carrier protein) reductase (fabG)	MkanA1_04087	$4e^{-23}$	Essential for type II fatty acid metabolism
1	Glucosyl transferase	MkanA1_02302	$1e^{-56}$	Carbohydrate metabolism
1	4'-phosphopantetheinyl transferase	MkanA1_00465	$4e^{-41}$	Aspartate aminotransferase family
1	Putative transferase	MkanA1_28691	$2e^{-16}$	Possible glucosyl transferase
1	ADP-ribosyltransferase	MkanA1_03662	$2e^{-33}$	Synthesis of second messenger cyclic ADP ribose
1	Oxidoreductase, fadB5	MkanA1_24990	$2e^{-22}$	Fatty acid degradation; energy production & conversion; general function prediction
1	NADH dehydrogenase subunit G	MkanA1_10142	$1e^{-21}$	NADH dehydrogenase/ubiquinone oxidoreductase
1	Para-aminobenzoate synthase component II	MkanA1_22545	$1e^{-26}$	Amino acid & folate metabolism
2	Glucose 1-phosphate cytidylyltransferase	MkanA1_11550	$6e^{-83}$	Nucleoside pyrophorylase, lipopolysaccharide biosynthesis & degradation of surface polysaccharides
2	XRE family transcription regulator (*M. gilvum* PYR-GCK)	Mflv_5549	$1e^{-51}$	Xenobiotic response element family of transcription regulators
19	No significant hits			

These putative proteins were obtained from a total of 45 ORFs. RND, Resistance, Nodulation & Division protein; ABC, ATP-Binding Cassette; MCE, Mammalian Cell Entry

Table 5.2: Putative ORFs (virulence genes) identified in *M. kansasii* subtype I showing sequence identity to known protein sequences in some pathogenic mycobacteria

ORF	Homologous Gene in other Pathogenic Mycobacteria	Gene Product	*E*-value	Sequence similarity (%)
MkanA1_02222	MUL_4809 (*M. ulcerans* Agy99)	O-methyltransferase	0.0	83
	MMAR_0313 (*M. marinum*)		0.0	83
	MAV_5200 (*M. avium* 104)		0.0	86
	MAB_3463 (*M. abscessus*)		$1e^{-44}$	70
MkanA1_09847	Rv2939 (*M. tuberculosis* H37Rv)	Acyltransferase (PapA5)	0.0	91
	Mb2964 (*M. bovis* AF2122/97)		0.0	91
	ML2349 (*M. leprae* TN)		0.0	90
	MMAR_1768 (*M. marinum*)		0.0	77
MkanA1_12468	MMAR_2472 (*M. marinum*)	Polyketide synthase (pks8)	0.0	86
	MAV_3108 (*M. avium* 104)		0.0	86
	Rv1661 (*M. tuberculosis* H37Rv)		0.0	86
	Mb1689 (*M. bovis* AF2122/97)		0.0	86
MkanA1_14385	MMAR_3025 (*M. marinum*)	Polyketide synthase (pks12)	0.0	93
	MUL_2266 (*M. ulcerans* Agy99)		0.0	93
	Mb2074c (*M. bovis* AF2122/97)		0.0	93
	MAV_2450 (*M. avium* 104)		0.0	91
MkanA1_24065	MAV_3356 (*M. avium* 104)	PPE Family Protein	$1e^{-30}$	79
	MMAR_2669 (*M. marinum*)		$7e^{-30}$	86
	Rv1808 (*M. tuberculosis* H37Rv)		$4e^{-29}$	89
	Mb1837 (*M. bovis* AF2122/97)		$4e^{-28}$	89
MkanA1_24070	MUL_3088 (*M. ulcerans* Agy99)	PE19_1	$6e^{-20}$	83
	Rv1788 (*M. tuberculosis* H37Rv)		$6e^{-20}$	84
	MMAR_2670 (*M. marinum*)		$6e^{-20}$	83
	Mb1816c (*M. bovis* AF2122/97)		$6e^{-20}$	83
MkanA1_27316	MUL_3292 (*M. ulcerans* Agy99)	PE-PGRS Family Protein	$5e^{-26}$	77
	MT0855 (*M. tuberculosis* CDC1551)		$8e^{-26}$	75
	Mb0857c (*M. bovis* AF2122/97)		$8e^{-26}$	75
	MMAR_2053 (*M. marinum*)		$1e^{-25}$	76
MkanA1_27376	MMAR_4705 (*M. marinum*)	MCE Family Protein	0.0	89
	MAP0765 (*M. avium* subsp. *paratuberculosis* K10)		0.0	87
	MUL_3923 (*M. ulcerans* Agy99)		0.0	82
	Rv1971 (*M. tuberculosis* H37Rv)		0.0	71
MkanA1_29416	Rv3595c (*M. tuberculosis* H37Rv)	PE-PGRS2	$6e^{-28}$	83
	Mb0857c (*M. bovis* AF2122/97)		$6e^{-28}$	83
	MMAR_3728 (*M. marinum*)		$2e^{-27}$	82
	MUL_3671 (*M. ulcerans* Agy99)		$3e^{-27}$	82
MkanA1_29646	MMAR_0299 (*M. marinum*)	PE-PGRS1	$2e^{-19}$	85
	MUL_0572 (*M. ulcerans* Agy99)		$2e^{-18}$	82
	Rv0834c (*M. tuberculosis* H37Rv)		$3e^{-18}$	80
	Mb0857c (*M. bovis* AF2122/97)		$3e^{-18}$	80

Proposed functions of these putative proteins are given above in Table 5.1 .

Amongst the ORFs detected were potential virulence factors, which included an *mce* gene (MkanA1_27376), five PE, PE-PGRS and PPE family genes, one methyltransferase gene, two polyketide synthase genes (*pks8* and *pks12*), an acyltransferase gene, *papA5* (Tables 5.1 and 5.2); three predicted drug exporters (MkanA1_19751 and MkanA1_02417); two transcription regulators involved in controlling levels of antibiotics (MkanA1_07199) and xenobiotics (Mflv_5549) in cells; putative glycosyltransferases and hydrolases involved in carbohydrate metabolism and transport (e.g, MkanA1_07384, MkanA1_02302, MkanA1_28691, MkanA1_11550); lipid metabolism and transport (e.g, MkaA1_09104, MkanA1_22222); and energy metabolism (e.g, MkanA1_24990) (Table 5.1). Other ORFs encoded oxidoreductases (MkanA1_24990, MkanA1_09190), dehydrogenases (e.g, MkanA1_21648, MkanA1_21648), isomerases (e.g, MkanA1_19761) and hydratases (e.g, MkanA1_03967) (Table 5.1).

5.4 Discussion

The *M. kansasii* species currently comprises seven 7 genetically very similar subtypes. Subtype I is considered to be the most pathogenic and commonly isolated subtype from humans infections. The other subtypes are mostly isolated from the environment, and are generally regarded to be non-pathogenic (Santin *et al*, 2004; Alcaide *et al*, 1997; Picardeau *et al*, 1997; Taillard *et al*, 2003). Due to their close relatedness, it is difficult to distinguish pathogenic subtype I from the other non-pathogenic subtypes. To identify differences between subtype I and the other subtypes, HMDA was used, a well-suited and cost-effective approach in such a case. The intent was not to exhaustively characterise all the genetic differences between the selected subtypes, but to determine if HMDA could isolate sequences divergent in subtype I.

Forty-five *M. kansasii* subtype I ORFs encoding predicted membrane-associated and secreted proteins were identified. Some of the ORFs obtained were those associated with virulence in other pathogenic mycobacterial species such as *M. marinum*, *M. ulcerans*, *M. tuberculosis* and *M. bovis*. These included *mce*, PE, PE-PGRS and PPE family genes, O-methyltransferase gene, an acyltransferase gene (*papA5*) and two polyketide synthase genes (*pks*8 and *pks*12). MCE (mammalian cell entry) proteins facilitate mycobacterial host cell invasion and intracellular cell survival (Arruda *et al*, 1993). Analysis of the draft genome sequence of *M. kansasii* ATCC 12478 (available on December 16, 2008 and last accessed on April 6, 2010) shows that it contains a number of putative *mce* genes. A recent study on actinomycetes has suggested that an increased number of *mce* operons may be associated with pathogenicity (Ishikawa *et al*, 2004). This may offer a significant advantage to pathogenic mycobacteria in their normal ecological niche (Romero and Palacios, 1997). The PE, PE-PGRS and PPE are gene families which possess

characteristic proline-glutamate (PE) or proline-proline-glutamate (PPE) residues at the N-terminus of their gene products (Cole *et al*, 1998; Brennan *et al*, 2001; Mackenzie *et al*, 2009). The other subgroup, PE-PGRS, contains multiple tandem repeats of glycine-glycine-alanine or glycine-glycine-asparagine encoded by genes with polymorphic GC-rich sequences (PGRS) motif (Poulet and Cole, 1995). These gene families are highly expanded in pathogenic members of mycobacteria but their functions are not clearly defined. They are often associated the ESX gene clusters, which encode ATP-dependent specific secretion systems (Cole *et al*, 1998; Gey van Pittius *et al*, 2006; Abdallah *et al*, 2009). Some members of this family have been found to be expressed on the cell surface (Brennan *et al*, 2001; Delogu *et al*, 2004), and to modulate immune responses (Choudhary *et al*, 2003; Basu *et al*, 2007; Abdallah *et al*, 2008). Studies in *M. marinum* have also suggested that they have a role in mycobacterial virulence (Brennan *et al*, 2001; Ramakrishnan *et al*, 2000).

O-methyltransferase and acyltransferase papA5 are part of the polyketide synthase biosynthetic pathway. O-methyltransferase is involved in the oxygenation of dimycocerosate (DIM) esters and phenolic glycolipids (PGL) (Onwueme *et al*, 2005a; Krzywinska *et al*, 2005; Ferreras *et al*, 2008; Huet *et al*, 2009), while acyltransferase papA5 is associated with the biosynthesis and transport of phthiocerol dimycocerosate (PDIM) and PGL (Buglino *et al*, 2004; Onwueme *et al*, 2004). The subtrates for these enzymes are major virulence factors of clinically relevant mycobacteria such as *M. kansasii* and *M. tuberculosis* (Onwueme *et al*, 2005b; Huet *et al*, 2009). The putative *pks8* and *pks12* genes products are members of polyketide synthases, a group of structurally and functionally diverse proteins important in the biosynthesis of complex mycobacterial lipids. Gene products of *pks8* and *pks12* are important in the generation of PDIM (Azad *et*

al, 1996; Sirakova *et al*, 2003; Cheng *et al*, 2009; Chopra and Gokhale, 2009). *M. kansasii* virulence factors have not been extensively studied, although it is known that some of its cell lipids modulate immune responses (Guerardel *et al*, 2003; Vignal *et al*, 2003; Fujita *et al*, 2007). All the genes described above may be important virulence factors contributing to the pathogenicity of *M. kansasii* subtype I. Elucidation of the potential role for these putative genes in the virulence of *M. kansasii* should be explored.

Bacteria generally respond to their changing environmental cues through their membrane proteins and DNA response elements. Identification of genes encoding transcription regulators and drug exporters from *M. kansasii* suggests that these systems may play a vital role in the environmental adaption of the bacterium to sites of infection in the host. TetR (name originally derived from *T*etracycline *R*esistance) family proteins are repressors that control the level of susceptibility to hydrophobic antibiotics and detergents (Aramaki *et al*, 1993; Aramaki *et al*, 1995; Ramos *et al*, 2005), while the XRE (xenobiotic response element) family proteins are responsible for controlling the levels of xenobiotics in the cell (Tran *et al*, 2005). Two other genes encoding multidrug efflux systems, a transmembrane transport protein (MmpL13_1) and a putative transmembrane ATP-binding protein (ABC-type transporter), were also detected. MmL13_1 (MmpL stands for *M*ycobacterial *m*embrane *p*rotein *L*arge) is member of the RND (*R*esistance, *N*odulation and Cell *D*ivision) protein family associated with multidrug resistance pumps, and are responsible for mediating the secretion of a diversity of compounds, including drugs, detergents and fatty acids (Poelarends *et al*, 2000; Putman *et al*, 2000a; Putman *et al*, 2000b). The role of MmL13_1 has not been confirmed, but some members of the RND family have been shown to be involved in the transport of PDIM (Cox *et al*, 1999; Camacho *et al*, 2001) and the synthesis of sulpholipid 1(Converse *et al*, 2003; Domenech

et al, 2005). Thus, this family of proteins may contribute to the virulence and drug resistance of mycobacteria (Domenech *et al*, 2005; Converse and Cox, 2005).

It was interesting to find that HMDA was also able to detect the presence of the *gyrA* gene in *M. kansasii* subtype I, and this raised the question as to whether the other three subtypes tested did not possess it. This gene is an important house-keeping gene in bacteria that encodes the A subunit of DNA gyrase, GyrA. The gene product is responsible for mediating double-strand breakage and reunion of DNA (Perler *et al*, 1994, *Nucleic Acid Res* 22: 1125-1127). Analysis of the *gyrA* locus from different mycobacterial species revealed the presence of unusual coding sequences inserted in-frame with a protein-coding sequence (Perler *et al*, 1994). The protein products of these sequences are referred to as inteins and are putative homing endonucleases. However, in genetically heterogeneous species such as *M. kansasii*, the presence and absence of intein-coding sequences has been reported (Fsihi *et al*, 1996; Sander *et al*, 1998). Investigation of the presence of these coding sequences in *gyrA* revealed that inteins were invariable associated with *M. kansasii* subtype I and not with *M. kansasii* subtype II strains (Sander *et al*, 1998). It is feasible that the presence of an intein-coding sequence in the *gyrA* of *M. kansasii* subtype I and its absence in the *gyrA* sequences of *M. kansasii* subtypes III, V and VI, explains the observation made in this study. To confirm this observation, sequence analysis of *gyrA* PCR amplification data from the *M. kansasii* subtypes has to be performed.

The identification of the large number of ORFs involved in lipid and carbohydrate metabolism and transport in this study suggests that most of these ORFs may participate in cell wall metabolism. This indicates that the major differences between subtype I and the other subtypes may lie in genes encoding cell wall components and some secreted

proteins. This difference might reflect that *M. kansasii* subtype I might have gained some additional genes during the course of its evolution (Koonin *et al*, 2001; Kinsella *et al*, 2003; Krzywinska *et al*, 2004)

5.5. Conclusion

Using HMDA, 45 ORFs divergent in *M. kansasii* subtype I and encoding predicted membrane proteins and secreted proteins were identified. The majority of the ORFs were predominantly genes predicted to encode for membrane proteins. Particularly striking was the identification of six ORFs encoding virulence-associated proteins. These virulence genes were also found to have orthologs in genomes of other pathogenic mycobacteria such as *M. tuberculosis*, *M. bovis*, *M. marinum* and *M. ulcerans*. These findings may support the notion that genes associated with host specificity are often involved in interactions involving distinct host cells, in modifying bacterial cell surface and in acquiring necessary nutrients from highly defined external sources. In addition, the genetic differences identified between the subtypes studied may indicate a process of diversifying the selection pressure to avoid host immune responses (Maiden *et al*, 1997; Selander *et al*, 1997; Tettellin *et al*, 2000).

Other genes detected included those encoding proteins involved in lipid and carbohydrate metabolism and transport, protein metabolism and transport, transcription regulators, efflux membrane pumps and those with unknown function. The HMDA technique used in this study represents one of the first attempts at identifying genetic differences between subtypes of *M. kansasii*.

Functional analysis of genes, especially those encoding proteins for virulence factors, identified by HMDA is expected to provide further insights into important aspects of host-pathogen interactions and pave a way for the development of diagnostic tests and therapeutic agents against *M. kansasii* disease. The functions of these genes can be investigated using a macrophage cell culture model and a murine infection model. First deletion mutants have to be created from *M. kansasii* subtype I, followed by the investigation of changes in phenotypes in a macrophage or mouse model (Pelicic *et al*, 1997; Silver *et al*, 1998).

CHAPTER 6

Modulatory Ability of *M. kansasii* on Human Lymphocyte Activity *In Vitro*

CHAPTER 6

Modulatory Ability of *M. kansasii* on Human Lymphocyte Activity *In Vitro*

6.0 Abstract

M. kansasii has been identified as an important emerging opportunistic pathogen (Griffith, 2002; Smith *et al*, 2003), but little is known about its actual pathogenesis. Recent data suggest that cell wall lipids of pathogenic mycobacteria modulate host immune responses (Jozefowski *et al*, 2008). The purpose of this study was to determine whether *M. kansasii* induces such immunomodulation on lymphocyte activity *in vitro*. T cell responses to cell wall lipids (Suppressor Cell-Activating Factor [SCAF]), generated by exposing monocytes to *M. kansasii* cell lysates, were assessed in a classical Lymphocyte Transformation (LT) assay and in a Cytometric Bead Array (CBA) assay. The LT assay showed marked suppression ($p<0.0001$) with all SCAFs from the six *M. kansasii* subtypes tested, which ranged from 37 to 51%. Subtype II showed the highest level of suppression, while subtype VI showed least suppression. In the CBA assay, of the Th2 cytokines, IL-4 was markedly increased, whilst the levels of IL-6 or IL-10 were not. The levels of two of the Th1 cytokines tested, IFN-γ and TNF-α were significantly suppressed by the SCAFs from three subtypes (I, II and III) tested ($p<0.0001$). Il-17A expression by MN cells exposed to SCAF was also dramatically and significantly reduced. Paradoxically, the expression levels of IL-2 were elevated in all the test samples. These results suggest that SCAF from *M. kansasii* modulates immune responses through suppression of lymphocyte blastogenesis and by altering the expression of Th1/Th2/Th17 cytokines by human lymphocytes *in vitro* for its own survival.

6.1 Introduction

The pathogenesis of *M. kansasii* is poorly understood, and unlike *M. tuberculosis*, very little information is available on the mechanisms of immune evasion and on what constitutes a protective immune response. In recent years there has been a lot of interest in understanding the role of cell wall lipids in mycobacterial disease. The mycobacterial cell wall is rich in lipids and polysaccharides (Lowary, 2001; Brennan, 2003), and some of these molecules are potent modulators of immunes response that arise from infection by these organisms (Briken *et al*, 2004; Karakousis *et al*, 2004; Manca *et al*, 2004). An important observation in patients with TB has been the *in vitro* dysfunction of circulating T cells. T cells from patients with active TB show altered proliferative responses when primed with *M. tuberculosis* antigens (Delgado *et al*, 2002; Chen *et al*, 2010). Other studies have also shown that activation and antigen presentation in *M. tuberculosis*-infected macrophages are strongly inhibited (Noss *et al*, 2001; Gehring *et al*, 2004; Chang *et al*, 2005; Pecora *et al*, 2006).

A number of mycobacterial lipids have been implicated in the modulation of $CD4^+$ T cell function. These molecules include lipoarabinomannans (LAM) and their biosynthetic precursors, lipomannans (LM), trehalose 6,6'-dimycolate (TDM, the cord factor), lipoproteins, phosphatidylinositol mannosides (PIM), phthiocerol dimycocerosate and sulpholipids (Guerardel *et al*, 2003; Vignal *et al*, 2003; Briken *et al*, 2004; Karakousis *et al*, 2004; Fujita *et al*, 2007; Jozefowski *et al*, 2008; Mahon *et al*, 2009). Some of these lipid molecules have been shown to be secreted by macrophages and affect the functioning of other immune cells in a paracrine manner (Wadee *et al*, 1983; Beatty *et al*, 2000; Beatty *et al*, 2001).

This immunomodulatory phenomenon induced by pathogenic mycobacteria on the host is complex and not well understood. It involves multiple mechanisms, including regulatory T cells, immunosuppressive cytokines and T cell anergy after prolonged exposure to mycobacterial antigens (Briken *et al*, 2004; Mahon *et al*, 2009; Sinsimer *et al*, 2010). Most of these mechanisms have been studied in TB but very little is known about how they operate in *M. kansasii* disease.

6.2 Objective of the Study

The objective of this study was to determine whether *M. kansasii* has the ability to induce immunomodulation of human lymphocyte activity *in vitro*. Such data is vital for the provision of insights into the pathogenic mechanisms mediated by *M. kansasii*, and eventually for paving a way in the development of a vaccine for controlling disease caused by *M. kansasii*.

6.3 Results

6.3.1 Experimental Design

An *in vitro* model developed by Wadee and co-workers (1983) for *M. tuberculosis* was used to aid the design of this study (Figure 6.1). Suppressor Cell-Activating Factor (SCAF) was produced from U937 cells, a human monocytic cell line, after exposure to *M. kansasii* subtypes I to VI cell sonicates. Mononuclear (MN) cells obtained from healthy donors were exposed to SCAF or control supernatants and its effects on MN cells were assessed by the Lymphocyte Transformation assay and by the Cytometric Bead Array (CBA) Th1/Th2/Th17 Cytokine Kit assay (BD Bioscience, San Diego, CA, USA). For experimental details refer to Material and Methods Section 2.7 in Chapter 2.

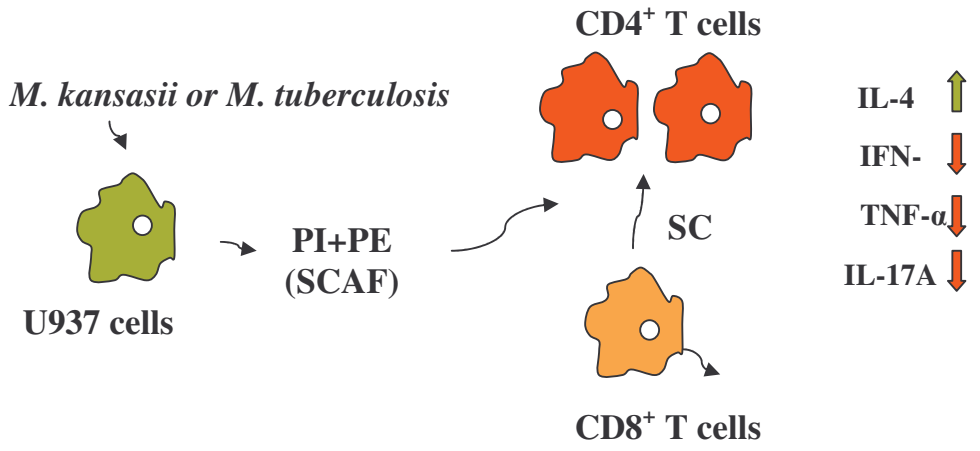


Figure 6.1: Experimental design. SCAF was produced by monocytes (U937 cells) in response to *M. kansasii* extracts (*M. tuberculosis* used as positive control). MN cells obtained from healthy donors were exposed to SCAF in the presence of ConA or PHA and lymphocyte blastogenesis or cytokine expression were assessed, respectively. SCAF, Suppressor Cell Activating Factor; SC, Suppressor Carbohydrates; PI, Phosphatidylinositol; PE, Phosphatidylethanolamine. Adapted from Smit van Dixhoorrn *et al*, 2008.

6.3.2 Effect of SCAF on Lymphocyte Blastogenesis

Studies have shown that SCAF generated from *M. tuberculosis* inhibits lymphocyte blastogenesis (Wadee *et al*, 1983). To determine whether SCAF from *M. kansasii* could induce similar inhibitory effects on lymphocyte proliferation, MN cells were stimulated with concanavalin A (Con A) for 72hrs in the presence or absence of SCAF. As shown in

Figure 6.2, SCAF from all six *M. kansasii* subtypes significantly ($p<0.0001$) inhibited the proliferation of lymphocytes. The levels of SCAF-mediated suppression ranged from 37 to 51% of unstimulated control, with subtype II showing the most suppression and subtype VI showing the least suppression (Table 6.1). These data indicate that SCAF generated from *M. kansasii* suppresses lymphocyte blastogenesis.

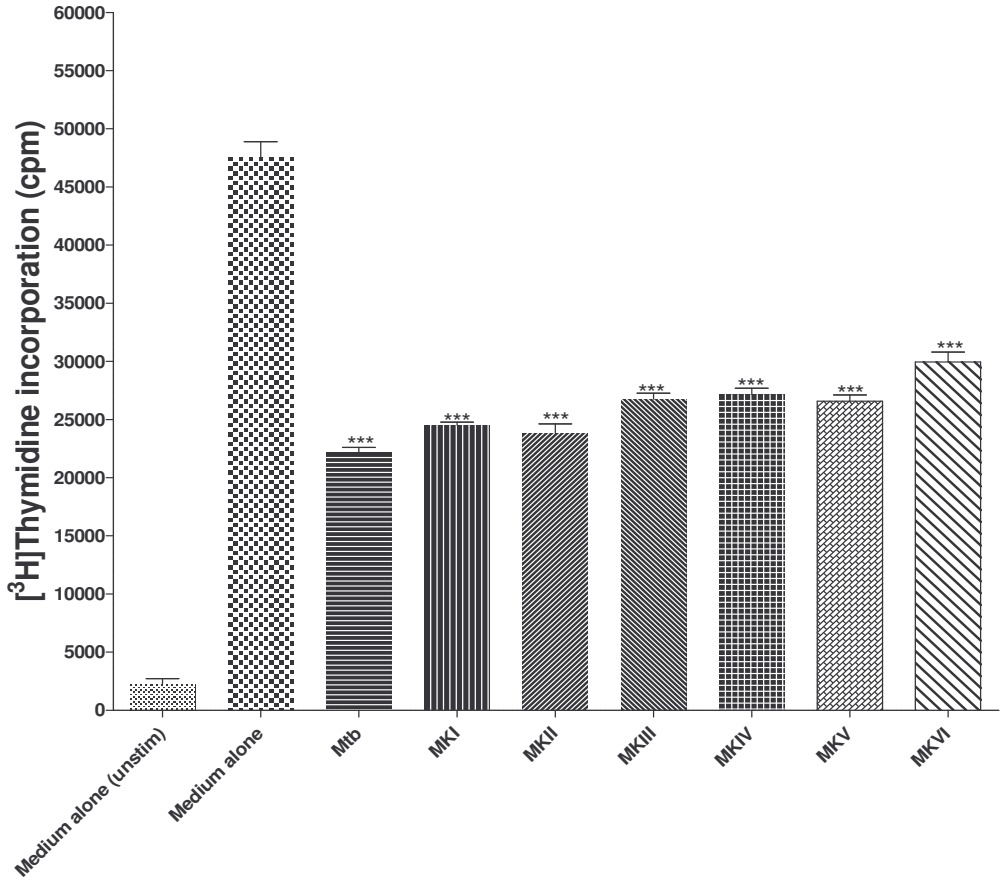


Figure 6.2: Effect of SCAF from *M. kansasii* subtypes I-VI on lymphocyte blastogenesis after 72hrs. Data are medians ± SEM of five independent experiments. P values were calculated using the Kruskal-Wallis test. The level of significance between the negative control (Medium alone) and the test samples was set at $p < 0.05$; cpm, counts per minute; Unstim, unstimulated; SCAF, Suppressor Cell Activating Factor; Mtb, *Mycobacterium tuberculosis*; MK, *Mycobacterium kansasii*

Table 6.1. Effect of SCAF from *M. kansasii* subtypes on the incorporation of tritiated thymidine in MN cells: suppression percentages

MN cells pulsed with	Subtype	Average cpm (n=5)	SD	Suppression (%)
M. tuberculosis H37Rv		22410	1608	51.5
M. kansasii	I	24840	980	46.3
	II	22840	2871	50.6
	III	28060	4369	39.3
	IV	26930	4113	41.8
	V	26370	2427	43.0
	VI	28880	3098	37.0
Medium alone + Con A		46270	4783	
Medium alone (unstimulated)		1873	1396	

The percentage of suppression for each condition was calculated as follows: ([MN cells treated with Medium alone] – [MN cells treated with SCAF]/[MN cells treated with Medium alone]) × 100%. Kruskal-Wallis test was performed to compare the SCAF-treated and Medium alone-treated samples. A significant (p<0.0001) level of SCAF-mediated suppression of lymphocyte blastogenesis was observed for all six *M. kansasii* subtypes tested. cpm, counts per minute; SCAF, Suppressor Cell Activating Factors; Con A, Concanavalin A..

6.3.3 Effect of SCAF on Cytokine Production

To determine whether the inhibitory effect observed in the Lymphocyte Transformation assay above could have been due to the suppression of Th1 and Th17 or promotion of Th2 T cell responses, levels of cytokines from these cells were measured in cell culture supernatants. Cytokine production by MN cells, obtained from seven healthy donors after exposure to SCAF generated from *M. tuberculosis* and *M. kansasii* subtypes I to III, was evaluated by the CBA Th1/Th2/Th17 Cytokine Kit assay that measures levels of IL-2, IL-4, Il-6, IL-10, IL-17A, IFN-γ and TNF-α. *M. tuberculosis* was used as a positive control, whereas Medium alone (MN cells + PHA + untreated culture supernatant) was used as a negative control. All values were calculated with regard to the negative control in each assay. The Kruskal-Wallis test, followed by the Dunn's multiple comparison adjustment test, was used for determining the statistical significance for the production of the cytokines.

The levels of IFN-γ, TNF-α and IL-17A were all observed to be significantly decreased for all the mycobacterial strains tested with regard to the negative control (all $p<0.0001$) (Figures 6.3A, B and G, respectively). The levels of suppression amongst the three *M. kansasii* subtypes were not significantly different from each other. The following were the medians for the three cytokines: IFN-γ (*M. tuberculosis*: 1879 pg/ml; *M. kansasii* subtype I-III: 2281, 2234 and 2356 pg/ml, respectively; negative control: 3925 pg/ml), TNF-α (*M. tuberculosis*: 273.9pg/ml; *M. kansasii* subtypes I-III: 305.2, 324.8 and 288.2pg/ml, respectively; negative control: 492.5pg/ml) and IL-17A (*M. tuberculosis*: 53.3pg/ml; *M. kansasii* subtypes I-III: 55.2, 59.3, and 60.9pg/ml, respectively; negative control: 172.8pg/ml). These data clearly indicate that SCAF from both *M. tuberculosis* and *M. kansasii* inhibits the production of signature cytokines of Th1 and Th17 T cells. In contrast to the other two Th1 cytokines, the levels of IL-2 were increased after exposure to all the SCAFs (Figure 6.3C).

SCAFs from all the four mycobacterial strains tested elicited significantly high levels of IL-4, which were more than double those of the negative control ($p<0.0001$) (Figure 6.3D) (*M. tuberculosis* median: 150.2 pg/ml; *M. kansasii* subtype I-III medians: 102.3, 88.8 and 119 pg/ml, respectively; negative control median: 36.8 pg/ml). The highest levels were observed with *M. tuberculosis* (four-fold increase), followed by subtype III, I and II, which showed at least a two-fold increase as compared to the negative control. In contrast, the levels of expression for the other two Th2 cytokines, IL-6 and IL-10, showed no statistically significant differences compared to the negative control ($p<0.9148$ and $p<0.9693$, respectively) (Figures 6.3E and F).

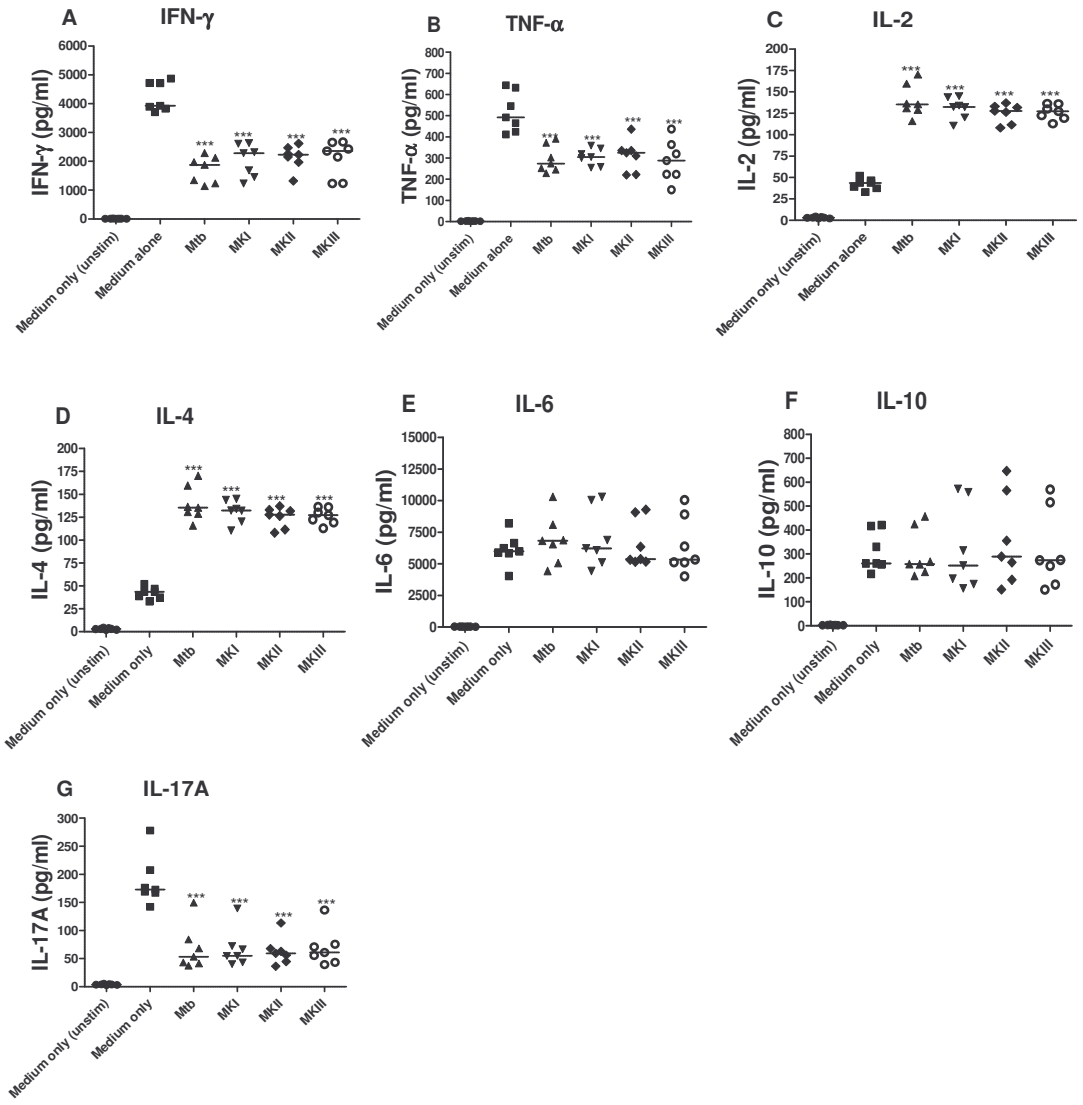


Figure 6.3. Cytokine production by MN cells after exposure to SCAF (*M. tuberculosis* or *M. kansasii*). Cytokine production by MN cells was stimulated for 72hrs in the presence of PHA and measured using the CBA Human Th1/Th2/Th17 Kit Assay. P values were calculated using the Kruskal-Wallis test. The level of significance between the negative control (Medium alone) and the test samples was set at $p < 0.05$. Unstim, unstimulated; SCAF, Suppressor Cell Activating Factor; Mtb, *Mycobacterium tuberculosis*; MK, *Mycobacterium kansasii*.

6.4 Discussion

One of the major thrusts in bacterial research is the understanding of the mechanisms responsible for pathogenesis. Lipids and glycolipids from pathogenic mycobacterial cell walls appear to be major molecules responsible for immune evasion (Guerardel *et al*, 2003; Vignal *et al*, 2003). Previous studies have demonstrated that human macrophages incubated with *M. tuberculosis* release mycobacterial lipids, termed SCAF, that are suppressive on $CD4^+$ T cell activity (Wadee *et al*, 1983). Since *M. kansasii* is one of the most virulent respiratory pathogens, it was postulated that it may also be employing its cell wall lipids to evade immune recognition through suppression of immune cells. The aim of the present study was undertaken not to completely explore the mechanism involved in immune suppression, but to extend the observations previously made with *M. tuberculosis* by examining the effects of SCAF from *M. kansasii* on the activation of MN cells obtained from a group of healthy donors. Its purpose was not to exhaustively analyse all the cytokines induced by *M. kansasii* but simply to provide preliminary data on the potential of *M. kansasii* to modulate immune responses. In line with previous findings above, SCAF from *M. kansasii* inhibited lymphocyte blastogenesis dramatically and modulated cytokine production from helper T cells significantly.

In the LT assay, inhibition of lymphocyte blastogenesis was observed with *M. tuberculosis* and all the *M. kansasii* subtypes tested (I-VI). This effect was also previously observed with *M. tuberculosis*, where the suppression was attributable to two lipid molecules, phosphatidylethanolamine (PE) and phosphatidylinositol (PI) (Wadee *et al*, 1983). Upon phagocytosis of mycobacteria, macrophages release PE and PI of bacterial origin into their

immediate surroundings, leading to the activation of $CD8^+$ T cells, which in turn produce another factor, termed Suppressor Carbohydrate (SC) (Sussman and Wadee, 1991; Smit van Dixhoorn *et al*, 2008). SC was shown to have the ability to suppress $CD4^+$ T cell activation as evidenced by the increased production of Th2 cytokines, and a drop in the expression levels of Th1 cytokines (Sussman and Wadee, 1991; Smit van Dixhoorn *et al*, 2008) (Figure 1). It is well-recognised that $CD4^+$ T cells and Th1 cytokines are critical in cell-mediated immune responses against mycobacterial infection (Flynn, 2004). In Th1 responses macrophages are activated by IFN-γ, which causes increased expression of inducible nitric oxide synthase (iNOS), indoleamine 2,3-deoxygenase, TNF-α and IL-6 (Voskuil *et al*, 2003; Pena *et al*, 2008). This activation process leads to the elimination of intracellular pathogens (Flynn *et al*, 1993; Szabo *et al*, 2003 Pena *et al*, 2008). Similarly, TNF-α plays a role in immunity against TB by helping to recruit immune cells to the site of infection and by inducing macrophage activation. TNF-α also enhances IFN-γ-induced production of reactive nitrogen intermediates and may, therefore, contribute to the killing of mycobacteria (Flynn *et al*, 1995; Ordway *et al*, 2004). We find that supernatants produced by U937 cells, pulsed with *M. kansasii* and *M. tuberculosis*, significantly abrogate the expression of IFN-γ and TNF-α by MN cells. This suggests that *M. kansasii* exploits the early stages of infection to drive cytokine expression to its own advantage, namely into a Th2 rather than into a self-destructive Th1 direction.

Data from other studies show that mycobacterial lipids suppress expression levels of IL-2 (Shabaana *et al*, 2005; Mahon *et al*, 2009; Palma-Nicolas *et al*, 2010). Thus, it was surprising to find that significantly increased levels of IL-2 were observed in this study following exposure of MN cells to SCAF. IL-2 is a T cell growth factor produced by $CD4^+$ effector T

cells to promote their proliferation and differentiation into antigen-specific T cells (Dooms *et al*, 2004). Recent evidence seems to suggest that distinct $CD4^+$ T cell subpopulations producing IL-2 and IFN-γ exist based on their expression of phenotypic surface markers and their ability to produce cytokines (Ahmed and Gray, 1996), and this may probably explain the incongruence observed in this study. IL-2 is predominantly produced by the long-lived (days) central memory T cells (T_{CM}), while IFN-γ is predominantly produced by the short-lived (hours) effector T cells (T_{EM}) (Sallusto *et al*, 1999; Lanzavecchia and Sallusto, 2000). A recent study showed that individuals with latent TB had higher frequencies of T_{CM} cells secreting IL-2 than those with active TB when exposed to purified peptide derivatives (PPD) or *M. bovis* BCG (Sargentini *et al*, 2009). This was attributed to an expanded population of IL-2-producing T_{CM} cells compared to the number of IFN-γ-producing T_{EM} cells which were reduced in these individuals, the absence of replicating *M. tuberculosis* and a small number of epitope-specific cells in the T_{CM} population (Millington *et al*, 2007; Sargentini *et al*, 2009). The IL-2 produced this way may be used for maintaining Tregs or Th2 cell population. It is feasible that the above phenomenon, i.e., the outgrowth of T_{CM} cells in our cultures and the autocrine production of Il-2 by effector CD4+ T cells in general, explains the overall increased expression in our study.

Increased Th2 responses have been postulated to play a role in the susceptibility to TB because cytokines such as IL-4 can suppress Th1-mediated immunity and drive inappropriate alternative activation of macrophages (Sallusto *et al*, 1999; Lanzavecchia and Sallusto, 2000). When macrophages are exposed to high levels of IL-4, an increased expression of arginase occurs (which hydrolyses L-arginine to urea and ornithine, the latter being important in

fibrosis) rather than that of iNOS (Millington *et al*, 2007; Sargentini *et al*, 2009). The observation made in our study in which expression levels of IL-4 were high and those of IFN-γ and TNF-α were low, match these observations.

Induction of Th17 responses to SCAF-treated MN cells was also examined. IL-17A, a pro-inflammatory cytokine produced by the recently described Th17 T cell subset (Harrington *et al*, 2006), was also suppressed by SCAF from all the mycobacterial strains tested. IL-17A is a potent inflammatory cytokine induced by *M. tuberculosis* (Khader and Cooper, 2008; Pasquinelli *et al*, 2009) and has been shown to up-regulate chemokines which led to increased recruitment of Th1 T cells (Khader *et al*, 2007) and facilitating granuloma formation (Umemura *et al*, 2007). Other data suggest that this suppression effect is mediated via RORγt, a Th17 cell differentiation transcription factor (Manel *et al*, 2008). Down-regulation of the IL-6 receptor (IL-6R) expression on $CD4^+$ T cells by *M. tuberculosis* may be a possible mechanism responsible for the observed suppression (Chen *et al*, 2010). Our experiment corroborates these findings, namely that *M. kansasii* significantly down-regulates IL-17A expression by MN cells, probably to escape a protective immune response.

The expression levels of IL-6 and IL-10 in response to SCAF were found to be non-specifically induced by PHA and not significantly different with regard to the negative control samples. IL-6 is a pro-inflammatory cytokine that is produced by a variety of cells such as T cells, macrophages, endothelial cells and fibroblasts. It plays a role in the initiation of T cell activation and has been shown to activate effector T cells to overcome immune suppression by $CD4^+CD25^+$ Tregs (Saunders and Cooper, 2000; Pasare and Medzhitov, 2003). IL-10 is

associated with reduced resistance and chronic progression of mycobacterial disease. It has multiple effects that interfere with functions of protective cells and cytokines, thereby promoting mycobacteria to survive inside cells despite abundant production of IFN-γ (Turner *et al*, 2002; De la Barrera *et al*, 2004; Rook, 2007). On the other hand, absence of IL-10 has been shown to accelerate mycobacterial clearance (van Crevel *et al*, 2002). Apparently, in our model *M. kansasii* and *M. tuberculosis* exert their immunomodulatory effects on cytokine expression through IL-4, IFN-γ, TNF-α, and IL-17A, rather than through IL-6 and IL-10.

A number of other lipids, including lipoarabimannan (LAM), from both *M. kansasii* (kanLAM, mannose-capped) and *M. tuberculosis* (ManLAM, mannose-capped) have also been implicated in immune cell modulation (Guerardel *et al*, 2003; Vignal *et al*, 2003; Briken *et al*, 2004). ManLAM has been shown to stimulate Antigen Presenting Cells (APC) through the mannose receptor, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), CD14, TLR-1 and TLR-2 in and on macrophages and dendritic cells to produce cytokines and chemokines that modulate the functioning of $CD4^+$ T cells (Chatterjee *et al*, 1992; Briken *et al*, 2004; Elass *et al*, 2007; Garg *et al*, 2008). Macrophages activated by ManLAM produce IL-10 and transforming growth factor-β (TGF-β), two of the cytokines contributing to the inhibition of Th1 T cells through the activation of regulatory T cells (Tregs) (Garg *et al*, 2008). More recently phosphatidylinositol mannosides (PIM), AraLAM (non-mannose-capped) and ManLAM were shown to directly inhibit $CD4^+$ T cell activation by interfering with the proximal T cell receptor (TCR) signalling and by abolishing IL-2 production (Mahon *et al*, 2009). It is thought that ManLAM is able to interfere with proximal TCR signalling by binding through the glycosylphatidylinositol (GPI) anchor in the plasma

membrane or through the lipid rafts of the $CD4^+$ T cells (Shabaana *et al*, 2005; Mahon *et al*, 2009). This recapitulates findings of earlier studies that also showed that binding of ManLAM to the lipid rafts on these cells leads to a reduction in the production of Th1 cytokines, IL-2 and IFN-γ and increased production of Th2 cytokines IL-4 and IL-5 (Shabaana *et al*, 2005).

Another glycolipid molecule, 2,3-di-O-acyl-trehalose (DAT) has also been shown to suppress the production of Th1 cytokines (Saavedra *et al*, 2001; 2006; Palma-Nicolas *et al*, 2010) through the down-regulation of the di-acyl-glycerol-dependent activation of the MAPK-ERK1/2 pathway (Palma-Nicolas *et al*, 2010). DAT is considered to be a mycobacterial virulence factor (Besra *et al*, 1992; Saavedra *et al*, 2006). Indeed these lipids are considered to be virulence factors, and we postulate that *M. kansasii* also mediates its exploitative effects on Th1/Th17 cytokine expression through these mechanisms. The consequence of this is a decline in protective immunity that results in the facilitation of the growth of the bacillus and disease progression.

However, our study has its limitations in that humoral and other cell mechanisms (e.g. cell-cell contact or secretion of other substances like perforin and granzymes) in addition to cytokine secretion or suppressor carbohydrates may also be involved in mechanisms of suppression but were not analysed. Recent insights into the possible mechanisms of immunosuppression suggest that galectins, a family of conserved glycan-binding proteins considered to play critical roles in the regulation of innate and adaptive immune responses (Barboni *et al*, 2005; Rabinovich *et al*, 2007; Yang *et al*, 2008; Lee *et al*, 2009), shift the balance from Th1 and Th17 towards a Th2-polarized immune response (Toscano *et al*, 2007;

Motran *et al*, 2008). In addition to the modulation of Th1 and 17 responses, galectins have been shown to favour the expansion of CD^+CD25^+ T regulatory cells (Toscano *et al*, 2006; Garin *et al*, 2007), arginase activity and prostaglandin E_2 production, and the inhibition of inducible nitric oxide synthase activity (Rangel-Moreno *et al*, 2002; Correa *et al*, 2003; Rabinovich *et al*, 2007). How these endogenous lectins selectively amplify Th2 and T regulatory cells remains to be explored.

6.5. Conclusion

This study demonstrates that *M. kansasii* adversely affects host immune responses through suppression of lymphocyte blastogenesis and inhibition of Th1 and Th17 T cell responses. These suppression effects, as evidenced by elevated expression levels of IL-4, a Th2 cytokine, and suppression of expression levels of Th1 cytokines, IFN-γ and TNF-α, and that of Th17 cytokine, IL-17A, are postulated to be mediated through cell wall lipids present in SCAF (Wadee *et al*, 1983). These effects may contribute to disease progression, especially in individuals whose immune responses are impaired such as those infected with HIV. Surprisingly, levels of IL-2, a Th1 cytokine, were elevated and this may suggest possible selection of T_{CM} cells amongst the T cell effector cells for maintenance of Tregs and/or Th2 cells.

Further work is required to identify the major components of SCAF responsible for the observed suppression effects and to unravel the molecular mechanisms responsible for these effects. The examination of the role of galectins in mycobacterial immune escape mechanisms is also warranted. This is critical for the better understanding of the interaction between *M.*

kansasii and the human immune system to facilitate the development of a vaccine against *M. kansasii* or other mycobacterial disease.

CHAPTER 7

General Conclusions and Future Work

CHAPTER 7
General Conclusions and Future Work

7.1 General Conclusions

M. kansasii has recently emerged as an important environmental opportunistic pathogen that causes both pulmonary and extrapulmonary disease in immunocompetent and immunosuppressed individuals (Campo and Campo, 1997; Taillard *et al*, 2003; Arend *et al*, 2004; Marras *et al*, 2004). Clinical and radiological features of *M. kansasii* pulmonary disease resemble those of tuberculosis (Field and Cowie, 2006; Shitrit *et al*, 2006; Cattamanchi *et al*, 2008). This may suggest that *M. kansasii* and *M. tuberculosis* share certain features such as virulence factors that are involved in their pathogenesis. While the sources of infection, genetic basis and pathogenic mechanisms have been widely studied in *M. tuberculosis*, little or nothing is known about them in *M. kansasii*. The work presented in this study makes some steps in contributing to the investigations of these problems.

The source of *M. kansasii* infection is an enigma. Available data in literature seem to suggest that water distribution systems are the main sources of *M. kansasii* infection (Engel *et al*, 1980; Kubin *et al*, 1980; Steadham, 1980; Chobot *et al*, 1997). Therefore, the need to confirm that water is the source of its infection is evident. The observation that *M. kansasii* is mainly isolated from water distribution systems necessitated the focus of the investigation of the source of infection in this study to be placed on water distribution systems in the two regions studied. Results presented in Chapter 3 clearly demonstrate that the water distribution systems in both the Vaal River gold-mining region and the Secunda coal-mining complex harbour a high NTM diversity that included both potentially pathogenic and non-pathogenic species.

Potentially pathogenic NTMs from the Vaal River gold-mining region included *M. kansasii*, *M. avium*, *M. fortuitum*, *M. peregrinum*, *M. chelonae*, *M. abscessus*, *M. parascrofulaceum*, *M. montefiorense* and the newly described species, *M. setense*, while the non-pathogenic species included *M. gordonae* and *M. goodii*. The predominant potentially pathogenic species from the Secunda coal-mining included *M. avium*, *M. intracellulare*, *M. peregrinum* and *M. chelonae* and *M. tusciae*, whilst the non-pathogenic ones included only *M. gordonae*. In agreement with other studies (Falkinham *et al*, 2001; Le Dantec *et al*, 2002; Falkinham *et al*, 2008; Feazel *et al*, 2009), the majority of the NTMs were isolated from showerhead biofilms. This finding is a very important public health issue as it suggests that showerheads pose a health risk to a lot of people, particularly to individuals with impaired immune responses (Le Dantec *et al*, 2002; Torvinen *et al*, 2004; Falkinham *et al*, 2008; Feazel *et al*, 2009).

There were five *M. kansasii* strains recovered from the Vaal River region, four of which were isolated from mine hostel showerheads and one from an underground pipe biofilm. They included subtypes I, IV and an isolate with a unique PRA pattern, probably representing a new subtype. No *M. kansasii* strain was isolated from the Secunda coal-mining complex. This contrasts some reports from Europe which have indicated that *M. kansasii* is prevalent in coal-mining regions (Kubin *et al*, 1980; Chobot *et al*, 1997).

Recent population genetics of *M. kansasii* have demonstrated that there is diversity amongst *M. kansasii* isolates recovered throughout the world and that subtype I is the predominant subtype recovered from clinical sources, while the other subtypes are generally isolated from the environment (Picardeau *et al*, 1997; Taillard *et al*, 2003; Zhang *et al*, 2004). No such

studies have been performed in South Africa despite the high incidence of *M. kansasii* disease reported from the gold mines. This warranted an investigation into the molecular epidemiology of the isolates from these mines. In line with other studies from other countries, this study showed that subtype I was also the predominant subtype amongst the clinical isolates (Chapter 4). Subtypes II and IV were also detected among the clinical isolates. One of the major findings was the detection of two sets of isolates with unique PRA patterns not previously reported, with one set giving a restriction pattern identical to that detected in an environmental isolate (Chapter 3). The detection of these two sets of unique isolates probably suggests the existence of new subtypes of *M. kansasii* in the Vaal River gold-mining region. Analysis of both clinical and environmental subtype I isolates generally showed that the isolates were genetically diverse in nature with clustering (Chapter 4). One of the three environmental subtype I isolates was found to be identical to a clinical isolate, suggesting that water distribution systems in the Vaal River gold-mining region are potential sources of *M. kansasii* infection for the gold-mining workforce (Chapters 3 and 4).

The genetic diversity pattern of subtype I isolates observed in this study contrasts that observed in studies from Japan, Europe and the United States, in which a tight clonal structure was observed within individual geographical regions (Iinuma *et al*, 1997; Picardeau *et al*, 1997; Zhang *et al*, 2004). This may be attributable to host risk factors such as HIV and silicosis that may influence the adaption of the strains in the susceptible miners or could be as a result of unique ecological factors in the South African gold-mining environment (Feil *et al*, 2001; Feil and Spratt, 2001; Coenye and LiPuma, 2003). To our knowledge this is the first

report from South Africa to document the isolation of *M. kansasii* from the environment and to show a clonal relationship between an environmental and a clinical isolate of *M. kansasii*.

However, this study had a number of limitations, including insufficient number of water samples and sites sampled, problems with fungal contamination of some cultures due to the lack of selective media and the slow growth of mycobacteria, and lack of patient data.

Since *M. kansasii* subtype I is regarded to be the most pathogenic subtype amongst the known seven subtypes (Alcaide *et al*, 1997; Taillard *et al*, 2003; Goy *et al*, 2007), it was of interest to identify genetic differences between this subtype and the other environmental isolates. Using a comparative genomic approach, it was hoped that subtype I unique or divergent sequences important for understanding its physiology, virulence and evolution would be identified (Boucher *et al*, 2001; McLeod *et al*, 2004). An application of such an approach to closely related microorganisms has revealed the presence of unique genes, some of which encode virulence factors (Kucerova *et al*, 2010). The majority of the ORFs identified in this study were predicted to encode membrane-associated proteins (Chapter 5). A total of 45 ORFs encoding predicted membrane and secreted proteins were identified that included 6 sets of putative virulence factors (MCE, two polyketide synthases, five PE family proteins, an acyltransferase, and an O-methyltransferase), 2 members of the transcription regulators for drug and xenobiotic metabolic pathway, 3 members of the multidrug efflux systems, a number of proteins associated with lipid metabolism and transport, carbohydrate metabolism and transport, and those with undefined function. The predominance of proteins associated with the bacterial membrane suggests that one of the major differences between subtype I and the

other subtypes is in the cell membrane. These results support findings from other studies in which large sequence differences in genes associated with membrane proteins have been reported in closely related organisms (Selander *et al*, 1997; Tarr *et al*, 2000; Tettellin *et al*, 2001; Shanks *et al*, 2006a). The observed genetic differences between closely related organisms may reflect a process of diversifying the selection pressure to avoid host immune responses, especially in pathogens (Maiden *et al*, 1997; Selander *et al*, 1997; Tettellin *et al*, 2000). To our knowledge this first time that differences between a pathogenic and non-pathogenic *M. kansasii* isolates have been shown at a genetic level.

The pathogenesis of *M. kansasii* is poorly understood. Recent immunological studies have demonstrated that *M. tuberculosis* and other pathogenic mycobacteria modulate immune responses through their cell wall lipids (Wadee *et al*, 1983; Elass *et al*, 2007; Garg *et al*, 2008; Mahon *et al*, 2009). However, little is known about the role of these lipids in *M. kansasii* disease. Previous studies have shown that when lymphocytes are exposed to SCAF (culture supernatant containing cell wall lipids) generated with *M. tuberculosis*, their proliferation is suppressed (Wadee *et al*, 1983). In this study SCAFs from 6 *M. kansasii* subtypes were also tested on human lymphocytes to determine whether they would induce a similar effect as that observed with *M. tuberculosis*. The results obtained in Chapter 6 clearly indicate that SCAFs from all the *M. kansasii* subtypes tested markedly suppressed lymphocyte blastogenesis, supporting earlier findings observed with *M. tuberculosis* (Wadee *et al*, 1983).

Further analysis of the SCAFs on $CD4^+$ T cell activity showed that they all suppressed the expression of protective Th1 signature cytokines, IFN-γ and TNF-α, and Th17 cytokine, IL-

17A, and promoted the expression of 'inappropriate' Th2 responses, indicated by the increased levels of IL-4 (Chapter 6). This was in-keeping with observations made in other studies (Rook *et al*, 2004; Manel *et al*, 2008; Mahon *et al*, 2009; Pasquinelli *et al*, 2009). Taken together, these data show that SCAF from *M. kansasii* suppresses lymphocyte blastogenesis and modulates the expression of cytokines from $CD4^+$ T cells *in vitro*. To the best of our knowledge, this is the first study to demonstrate that *M. kansasii* suppresses lymphocyte proliferation and modulates cytokine expression from $CD4^+$ T cells through suppression of Th1 and Th17 cell responses, and promotion of "inappropriate" Th2 cell responses.

This PhD study has demonstrated that water distribution systems are the potential sources of *M. kansasii* and other mycobacterial infections for the miners in the Vaal River gold-mining region and that subtype I is the predominant clinical isolate of *M. kansasii* subtype in this region. In contrast to the clonal population structure of subtype I isolates within individual geographical regions of other parts of the world, DNA fingerprinting of subtype I isolates in this region were found to be genetically diverse. It also showed that probably two other subtypes of *M. kansasii* not detected in other regions of the world are present in the South African environment and that the major differences between subtype I and the other subtypes lie in genes encoding cell membrane proteins, some of which are virulence factors. It goes on to demonstrate that one of the major pathogenic mechanisms for *M. kansasii* is through the suppression of human lymphocyte activity. To our knowledge this the first study to demonstrate that water distribution systems in South Africa are possible sources of *M. kansasii* infection and to employ molecular tools to identify different genotypes of this

pathogen, to identify unique or divergent genes in subtype I, and to demonstrate that immunosuppression is one of the pathogenic mechanisms employed by *M. kansasii*.

7.2 Future Work

Following the investigations described in this thesis, several lines of research should be pursued. With the potential for *M. kansasii* infections resulting from water distribution systems, it would be important to examine factors that influence the survival and growth of *M. kansasii* in these systems. This can be achieved by examining the role of factors such as nutrient levels, pipe materials and disinfection on the survival of *M. kansasii* in model drinking water distribution system biofilms. This would help in providing useful information on the survival of the organism and the basis for identifying means of control of infections.

It would also be of interest to develop an infection model to demonstrate the transmission of *M. kansasii* from water to humans. Aerosolisation of water mycobacteria under conditions designed to mimic activities of a person taking a shower or swimming can be tested with the aid of a loaded six-stage Andersen Cascade Sampler (Andersen, 1958) and a suitable murine infection model. This would aid in establishing that water is a definitive source of *M. kansasii* infection in the Vaal River gold-mining region.

Further studies on the distribution of *M. kansasii* strains from other parts of South Africa should be performed to give an accurate picture of the *M. kansasii* strains circulating in the country. This should include both environmental and clinical isolates of *M. kansasii*.

It would also be of interest to perform functional analysis on genes identified by HMDA, especially those predicted to encode virulence factors. This can be investigated by first deleting the genes identified, followed by assessing changes in the resulting mutant phenotypes in a macrophage or mouse model (Pelicic *et al*, 1997; Silver *et al*, 1998). This would provide insights into important aspects of host-pathogen interactions, and pave a way for the development of diagnostic tests and therapeutic against *M. kansasii* disease.

The identification of major components of SCAF responsible for the observed suppressive and immunomodulative effects and the unravelling of the molecular mechanism for these effects would be of paramount importance. A good approach of achieving this would be to isolate and identify the lipids in SCAF using methods such as Gas Chromatography-Mass Spectroscopy (GC-MS) (Slayden and Barry, 2001). The effects of the isolated lipids can then be confirmed by testing in an LT assay, by cytokine analysis and by microarray analysis (Smit van Dixhoorn *et al*, 2008). This is vital in the facilitation of the development of a vaccine against *M. kansasii* or other NTM disease.

APPENDICES

Appendix I: Ethics Approval Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Kwenda

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M050707**
PROJECT	Molecular Characterisation of Clinical and Environmental Isolates of Mycobacterium kansasii in South Africa
INVESTIGATORS	Mr G Kwenda
DEPARTMENT	Clinical Microbiology & Infec. Dis
DATE CONSIDERED	05.07.29
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 05.08.01 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr E Marais

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix II: Identification of NTMs from water and biofilm samples obtained from the Vaal River gold-mining region and the Secunda coal-mining complex water distribution systems

				Analysis Results						
Region	Sample ID	Sample	Site Description	Raw Sample		Culture		Species		
				ZN	MK	PCR		ZN	MK	PCR	Isolated
VMR	NCHSH	Biofilm	Change house showerhead	-	-	+	+				*M. kansasii*
	LRR	Biofilm	Hostel showerhead	-	-	+	+				*M. kansasii*
	LRRSH-1	Biofilm	Hostel showerhead	-	-	+	+				*M. kansasii*
	WSQR	Biofilm	Hostel showerhead	-	-	+	+				*M. kansasii*
	KPUGW-I	Biofilm	Mine underground pipe	-	-	+	+				*M. kansasii*
	NRR	Biofilm	Hostel tap	-	-	+	-				*M. avium*
	WVHXD	Biofilm	Hospital X-ray Dept tap	-	-	+	-				*M. avium*
	LRK	Biofilm	Hostel kitchen tap	-	-	+	-				*M. avium*
	NRK	Biofilm	Hostel kitchen tap	-	-	+	-				*M. avium*
SMC	BCHSH	Biofilm	Change house showerhead	-	-	+	-				*M. avium*
	BCH	Biofilm	Change house showerhead	-	-	+	-				*M. avium*
	MCHSH	Biofilm	Change house showerhead	-	-	+	-				*M. intracellulare*
VMR	WVHDW	Water	Hospital ward tap	-	-	+	-				*M. fortuitum*
	KRKW	Water	Hostel kitchen tap	-	-	+	-				*M. fortuitum*
	NRT14	Water	Hostel tap	-	-	+	-				*M. fortuitum*
	KTR	Biofilm	Taxi rank public tap	-	-	+	-				*M. fortuitum*
	IRK	Biofilm	Hostel kitchen tap	-	-	+	-				*M. fortuitum*
	NHT-1	Biofilm	Hostel tap	-	-	+	-				*M. fortuitum*
	NFT22	Water	Hostel tap	-	-	+	-				*M. fortuitum*
	NKT	Water	Hostel kitchen tap	-	-	+	-				*M. peregrinum*
	KRK	Biofilm	Hostel kitchen tap	-	-	+	-				*M. peregrinum*
SMC	BHBTW	Biofilm	Hostel tap	-	-	+	-				*M. peregrinum*
	MCH-2	Biofilm	Change house showerhead	-	-	+	-				*M. peregrinum*
	MCHTW-1	Biofilm	Change house tap	-	-	+	-				*M. peregrinum*
	BCHTW	Biofilm	Hostel showerhead	-	-	+	-				*M. chelonae*
	SSC	Biofilm	Office kitchen tap	-	-	+	-				*M. chelonae*
	MCH-1	Biofilm	Change house showerhead	-	-	+	-				*M. chelonae*
	BHBSW	Biofilm	Hostel showerhead	-	-	+	-				*M. chelonae*
VMR	MFW	Biofilm	Hostel tap	-	-	+	-				*M. chelonae*
	LCH	Biofilm	Change house showerhead	-	-	+	-				*M. chelonae*
	KFW	Biofilm	Hostel tap	-	-	+	-				*M. chelonae*
	KRWCH	Biofilm	Hostel showerhead	-	-	+	-				*M. parascrofulaceum*
	UMQ	Biofilm	Hostel tap	-	-	+	-				*M. gordonae*
	SWSH	Biofilm	Hospital ward showerhead	-	-	+	-				*M. gordonae*
	HKT	Biofilm	Hostel kitchen tap	-	-	+	-				*M. gordonae*
	ESQK	Water	Hostel kitchen tap	-	-	+	-				*M. gordonae*
	KRCP	Biofilm	Hostel tap	-	-	+	-				*M. gordonae*
	NCH-1	Water	Change house showerhead	-	-	+	-				*M. gordonae*
	KKT	Biofilm	Hostel kitchen tap	-	-	+	-				*M. gordonae*
	LRRT	Biofilm	Hostel tap	-	-	+	-				*M. gordonae*
	LRRSH-2	Biofilm	Hostel showerhead	-	-	+	-				*M. gordonae*
	NCH-2	Biofilm	Change house tap	-	-	+	-				*M. gordonae*
	NHT-1	Biofilm	Hostel tap	-	-	+	-				*M. gordonae*
	MQB7	Biofilm	Hostel tap	-	-	+	-				*M. gordonae*

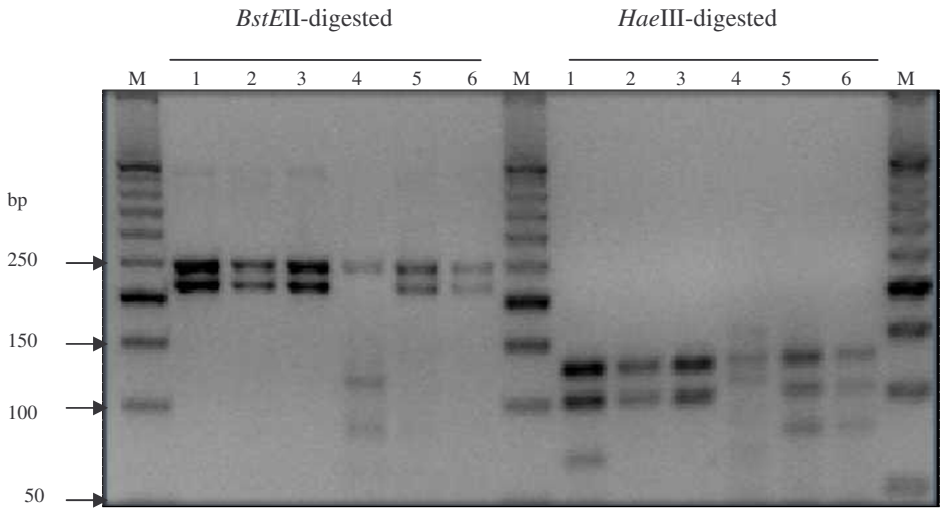
Continued on next page

Appendix II continued from previous page

SMC	BHBTS	Biofilm	Hostel tap	-	-	+	-	*M. gordonae*
	MCHTW-2	Water	Change house tap	-	-	+	-	*M. gordonae*
	BHB	Biofilm	Hostel tap	-	-	+	-	*M. gordonae*
	BHK	Biofilm	Hostel tap	-	-	+	-	*M. gordonae*
	BHKW	Biofilm	Hostel kitchen tap	-	-	+	-	*M. gordonae*
	C5WTS	Biofilm	Mine surface tap	-	-	+	-	*M. gordonae*
	SP6PS	Biofilm	Mine waste pipe	-	-	+	-	*M. tuscae*
VMR	NHT-2	Biofilm	Hostel tap	-	-	+	-	*M. goodii*
	NDH	Water	Dining hall tap	-	-	+	-	*M. montefiorense*
	UTS	Biofilm	Township public tap	-	-	+	-	*M. setense*
	KRSH	Biofilm	Hostel showerhead	-	-	+	-	*M. abscessus*

ZN, Ziehl-Neelsen; SMC, Secunda Coal-Mining Complex; VMR, Vaal River Gold Mining Region; MK PCR, *M. kansasii*-specific PCR

Appendix III: PRA patterns of representative clinical and environmental isolates of *M. kansasii*.



NB: Numbers above the lanes represent isolates and M denotes the molecular weight marker. Lane M, 50bp DNA marker (Fermentas Life Sciences, Glen Burnie, MD, USA); lane 1, subtype 1 (clinical isolate, MK087); lanes 2 and 3, unique isolate 2 (clinical isolates MK044 and MK136; not previously reported, probably new subtype); lane 4, subtype IV (environmental isolate LRR); lane 5 and 6, subtype I (environmental isolates KPUGW and WSQR). bp, base pair.

Appendix IV: Identification by DNA sequencing of two representative *M. kansasii* clinical isolates not showing the typical *M. kansasii* PRA patterns (unique isolates 1 and 2, Chapter 4)

Two genes, *secA*1 and *gyrB*, and the spacer sequence, 16S-23S rRNA ITS region, were targeted for DNA sequencing to confirm the identity of the clinical isolates with unique PRA patterns. Both isolates were confirmed to be *M. kansasii* as shown below.

1. Unique Isolate 1 (MK057)
a) *secA*1 gene sequencing

gi|57340229|gb|AY724717.1| *Mycobacterium kansasii* strain ATCC 12478 preprotein translocase SecA1 (secA1) gene, partial cds
Length=700; Score = 1231 bits (640), Expect = 0.0; Identities = 640/640 (100%), Gaps = 0/640 (0%); Strand=Plus/Plus

```
Query  1    GGCCTACAACGCCGACATCACCTACGGCACCAACAACGAGTTCGGCTTCGACTACCTGCG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GGCCTACAACGCCGACATCACCTACGGCACCAACAACGAGTTCGGCTTCGACTACCTGCG  120

Query  61   CGACAACATGGCGCACTCGCTCGACGACCTGGTGCAGCGCGGGCACGACTTCGCCATCGT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CGACAACATGGCGCACTCGCTCGACGACCTGGTGCAGCGCGGGCACGACTTCGCCATCGT  180

Query  121  CGACGAGGTCGACTCCATCCTGATCGACGAGGCCCGCACCCCGCTGATCATCTCCGGCCC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  CGACGAGGTCGACTCCATCCTGATCGACGAGGCCCGCACCCCGCTGATCATCTCCGGCCC  240

Query  181  CGCCGACGGCGCCTCCAACTGGTACGTCGAGTTCGCCCGGCTGGCGCCGCTGATGGAAAA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CGCCGACGGCGCCTCCAACTGGTACGTCGAGTTCGCCCGGCTGGCGCCGCTGATGGAAAA  300

Query  241  GGACACCCACTACGAGGTAGACCTGCGTAAACGCACCGTCGGCGTGCACGAGAAGGGCGT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GGACACCCACTACGAGGTAGACCTGCGTAAACGCACCGTCGGCGTGCACGAGAAGGGCGT  360

Query  301  GGAGTTCGTCGAAGACCAGCTCGGTATCGACAACCTCTACGAGGCCGCCAACTCGCCGCT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GGAGTTCGTCGAAGACCAGCTCGGTATCGACAACCTCTACGAGGCCGCCAACTCGCCGCT  420

Query  361  GGTCAGCTACCTCAACAATGCGCTGAAGGCCAAGGAGCTGTTCAACCGCGACAAGGACTA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GGTCAGCTACCTCAACAATGCGCTGAAGGCCAAGGAGCTGTTCAACCGCGACAAGGACTA  480

Query  421  CATCGTCCGCGACGGCGAGGTGCTCATCGTCGACGAGTTCACCGGTCGGGTGCTCTACGG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  CATCGTCCGCGACGGCGAGGTGCTCATCGTCGACGAGTTCACCGGTCGGGTGCTCTACGG  540

Query  481  GCGCCGCTACAACGAGGGCATGCACCAGGCCATCGAGGCCAAGGAGCACGTCGAGATCAA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  GCGCCGCTACAACGAGGGCATGCACCAGGCCATCGAGGCCAAGGAGCACGTCGAGATCAA  600

Query  541  GGCCGAGAACCAGACGCTGGCCACCATCACGCTGCAGAACTACTTCCGGCTCTACGACAA  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  GGCCGAGAACCAGACGCTGGCCACCATCACGCTGCAGAACTACTTCCGGCTCTACGACAA  660

Query  601  GCTCGCCGGCATGACCGGCACCGCCCAGACCGAGGCGGCC  640
            ||||||||||||||||||||||||||||||||||||||||
Sbjct  661  GCTCGCCGGCATGACCGGCACCGCCCAGACCGAGGCGGCC  700
```

b) *gyrB* gene sequencing

gi|6729314|dbj|AB014307.1| *Mycobacterium kansasii gyrB* gene for DNA gyrase B subunit, partial cds, strain KPM 1988-1
Length=1257; Score = 1297 bits (702), Expect = 0.0; Identities = 702/702 (100%), Gaps = 0/702 (0%); Strand=Plus/Plus

```
Query  2    TTACGAGAAATCCGAGCCGATGGGACTCAAGCAAGGCGCGCCGACTAAGAAGACCGGCAC  61
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  117  TTACGAGAAATCCGAGCCGATGGGACTCAAGCAAGGCGCGCCGACTAAGAAGACCGGCAC  176

Query  62   GACGGTGCGGTTCTGGGCCGATCCCAATGTTTTTGAGACCACCGAGTACGACTTCGAAAC  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  177  GACGGTGCGGTTCTGGGCCGATCCCAATGTTTTTGAGACCACCGAGTACGACTTCGAAAC  236

Query  122  CGTCGCACGACGGTTGCAGGAGATGGCGTTTCTCAACAAGGGGCTCACCATCAATCTGAC  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  237  CGTCGCACGACGGTTGCAGGAGATGGCGTTTCTCAACAAGGGGCTCACCATCAATCTGAC  296

Query  182  CGATCAGCGGGTGACCCAGGACGAGGTCGTCGACGAGGTGGTCAGCGACGTCGCCGAGGC  241
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  297  CGATCAGCGGGTGACCCAGGACGAGGTCGTCGACGAGGTGGTCAGCGACGTCGCCGAGGC  356

Query  242  CCCAAAGTCGGCCAGCGAGAAGGCGGCCGAATCCGCCGCCCCGCACAAGGTCAAGAAGCG  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357  CCCAAAGTCGGCCAGCGAGAAGGCGGCCGAATCCGCCGCCCCGCACAAGGTCAAGAAGCG  416

Query  302  TACCTTCCACTATCCCGGGGGTCTGGTTGACTTCGTCAAGCACATCAACCGGACCAAGAA  361
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  417  TACCTTCCACTATCCCGGGGGTCTGGTTGACTTCGTCAAGCACATCAACCGGACCAAGAA  476

Query  362  CGCCATCCACAGCAGCATCGTCGACTTCTCCGGTAAGGGACCCGGCCACGAAGTGGAGAT  421
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  477  CGCCATCCACAGCAGCATCGTCGACTTCTCCGGTAAGGGACCCGGCCACGAAGTGGAGAT  536

Query  422  CGCGATGCAGTGGAATGCCGGCTATTCGGAGTCGGTGCATACCTTCGCCAACACCATCAA  481
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  537  CGCGATGCAGTGGAATGCCGGCTATTCGGAGTCGGTGCATACCTTCGCCAACACCATCAA  596

Query  482  CACCCACGAGGGTGGGACCCACGAAGAGGGGTTCCGCAGCGCGCTGACCTCGGTGGTGAA  541
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  597  CACCCACGAGGGTGGGACCCACGAAGAGGGGTTCCGCAGCGCGCTGACCTCGGTGGTGAA  656

Query  542  CAAGTACGCCAAGGACCGCAAACTGCTCAAGGAAAAGGACCCCAACCTCACCGGCGACGA  601
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  657  CAAGTACGCCAAGGACCGCAAACTGCTCAAGGAAAAGGACCCCAACCTCACCGGCGACGA  716

Query  602  CATCCGGGAAGGGTTGGCCGCGGTGATTTCGGTCAAGGTCAGCGAGCCGCAGTTCGAGGG  661
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  717  CATCCGGGAAGGGTTGGCCGCGGTGATTTCGGTCAAGGTCAGCGAGCCGCAGTTCGAGGG  776

Query  662  CCAGACCAAGACGAAACTGGGCAACACCGAGGTGAAGTCGTT  703
            |||||||||||||||||||||||||||||||||||||||||||
Sbjct  777  CCAGACCAAGACGAAACTGGGCAACACCGAGGTGAAGTCGTT  818
```

c) 16S-23S rRNA ITS region sequencing

gi|146218732|emb|AM709725.1| *Mycobacterium kansasii* partial 16S rRNA gene, partial 23S rRNA gene and ITS1, isolate K7377-03
Length=404; Score = 582 bits (315), Expect = 3e-163; Identities = 315/315 (100%), Gaps = 0/315 (0%); Strand=Plus/Plus

```
Query  1    GCATCCCAACAAGTGGGGTGCAAGCCGTGAGGGGTTCTCGTCTGTAGTGGACGAAAGCCG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  81   GCATCCCAACAAGTGGGGTGCAAGCCGTGAGGGGTTCTCGTCTGTAGTGGACGAAAGCCG  140

Query  61   GGTGCACGACAACAAGCAAAGCCAGACACACTATTGGGTCCTGAGGCAACACTCGGGCTC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  141  GGTGCACGACAACAAGCAAAGCCAGACACACTATTGGGTCCTGAGGCAACACTCGGGCTC  200

Query  121  TGTTCGAGAGTTGTCCCACCATCTTGGTGGTGGGGTGTGGTGTTTGAGAATTGGATAGTG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201  TGTTCGAGAGTTGTCCCACCATCTTGGTGGTGGGGTGTGGTGTTTGAGAATTGGATAGTG  260

Query  181  GTTGCGAGCATCAAATGGATGCGTTGCCCTACGGGTAGCGTGTTCTTTTGTGCAATTTTA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261  GTTGCGAGCATCAAATGGATGCGTTGCCCTACGGGTAGCGTGTTCTTTTGTGCAATTTTA  320

Query  241  TTCTTTGGTTTTTGTGTTTGTAAGTGTCTAAGGGCGCATGGTGGATGCCTTGGCATCGAG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  321  TTCTTTGGTTTTTGTGTTTGTAAGTGTCTAAGGGCGCATGGTGGATGCCTTGGCATCGAG  380

Query  301  AGCCGATGAAGGACG  315
            |||||||||||||||
Sbjct  381  AGCCGATGAAGGACG  395
```

2. Unique isolate 2 (MK136)
a) *secA*1 gene sequencing

gi|57340229|gb|AY724717.1| *Mycobacterium kansasii* strain ATCC 12478 preprotein translocase SecA1 (secA1) gene, partial cds
Length=700; Score = 599 bits (324), Expect = 3e-168; Identities = 324/324 (100%), Gaps = 0/324 (0%); Strand=Plus/Plus

```
Query  1    CAGCGCCGGGTGGCCTACAACGCCGACATCACCTACGGCACCAACAACGAGTTCGGCTTC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  50   CAGCGCCGGGTGGCCTACAACGCCGACATCACCTACGGCACCAACAACGAGTTCGGCTTC  109

Query  61   GACTACCTGCGCGACAACATGGCGCACTCGCTCGACGACCTGGTGCAGCGCGGGCACGAC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  110  GACTACCTGCGCGACAACATGGCGCACTCGCTCGACGACCTGGTGCAGCGCGGGCACGAC  169

Query  121  TTCGCCATCGTCGACGAGGTCGACTCCATCCTGATCGACGAGGCCCGCACCCCGCTGATC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  170  TTCGCCATCGTCGACGAGGTCGACTCCATCCTGATCGACGAGGCCCGCACCCCGCTGATC  229

Query  181  ATCTCCGGCCCCGCCGACGGCGCCTCCAACTGGTACGTCGAGTTCGCCCGGCTGGCGCCG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  230  ATCTCCGGCCCCGCCGACGGCGCCTCCAACTGGTACGTCGAGTTCGCCCGGCTGGCGCCG  289

Query  241  CTGATGGAAAAGGACACCCACTACGAGGTAGACCTGCGTAAACGCACCGTCGGCGTGCAC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  290  CTGATGGAAAAGGACACCCACTACGAGGTAGACCTGCGTAAACGCACCGTCGGCGTGCAC  349

Query  301  GAGAAGGGCGTGGAGTTCGTCGAA  324
            ||||||||||||||||||||||||
Sbjct  350  GAGAAGGGCGTGGAGTTCGTCGAA  373
```

b) *gyrB* gene sequencing

gi|6729314|dbj|AB014307.1| *Mycobacterium kansasii gyrB* gene for DNA gyrase B subunit, partial cds, strain KPM 1988-1
Length=1257; Score = 1604 bits (868), Expect = 0.0; Identities = 870/871 (99%), Gaps = 0/871 (0%); Strand=Plus/Plus

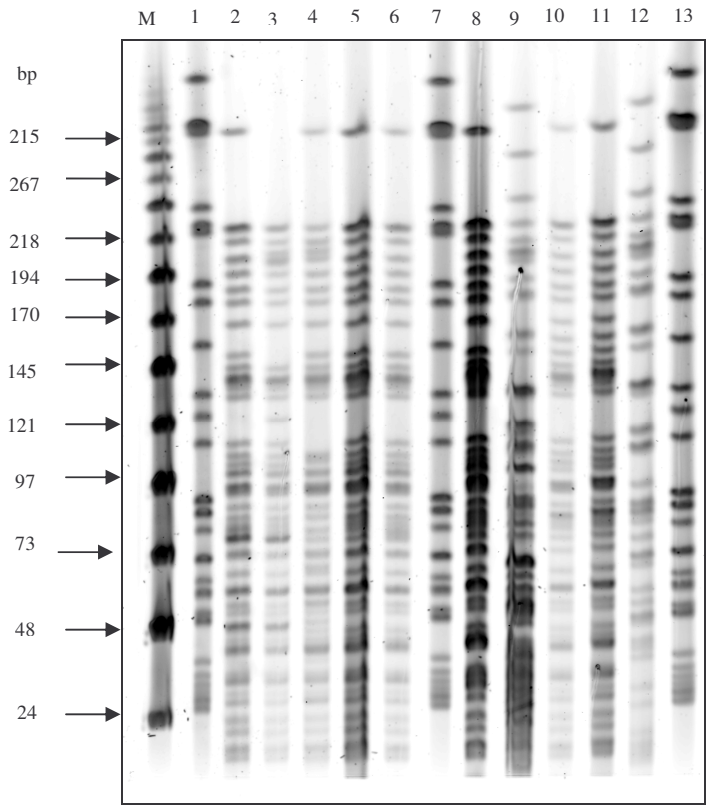
```
Query  2    ACGCACTGTCCACCCGGCTGGAGGTGGAGATCAAGCGCGACGGCCATGAGTGGTCGCAGG  61
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  56   ACGCACTGTCCACCCGGCTGGAGGTGGAGATCAAGCGCGACGGCCATGAGTGGTCGCAGG  115

Query  62   TTTACGAGAAATCCGAGCCGATGGGACTCAAGCAAGGCGCGCCGACTAAGAAGACCGGCA  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  116  TTTACGAGAAATCCGAGCCGATGGGACTCAAGCAAGGCGCGCCGACTAAGAAGACCGGCA  175

Query  122  CGACGGTGCGGTTCTGGGCCGATCCCAATGTTTTTGAGACCACCGAGTACGACTTCGAAA  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  176  CGACGGTGCGGTTCTGGGCCGATCCCAATGTTTTTGAGACCACCGAGTACGACTTCGAAA  235

Query  182  CCGTCGCACGACGGTTGCAGGAGATGGCGTTTCTCAACAAGGGGCTCACCATCAATCTGA  241
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  236  CCGTCGCACGACGGTTGCAGGAGATGGCGTTTCTCAACAAGGGGCTCACCATCAATCTGA  295

Query  242  CCGATCAGCGGGTGACCCAGGACGAGGTCGTCGACGAGGTGGTCAGCGACGTCGCCGAGG  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  296  CCGATCAGCGGGTGACCCAGGACGAGGTCGTCGACGAGGTGGTCAGCGACGTCGCCGAGG  355

Query  302  CCCCAAAGTCGGCCAGCGAGAAGGCGGCCGAATCCGCCGCCCCGCACAAGGTCAAGAAGC  361
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  356  CCCCAAAGTCGGCCAGCGAGAAGGCGGCCGAATCCGCCGCCCCGCACAAGGTCAAGAAGC  415

Query  362  GTACCTTCCACTATCCCGGGGGTCTGGTTGACTTCGTCAAGCACATCAACCGGACCAAGA  421
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  416  GTACCTTCCACTATCCCGGGGGTCTGGTTGACTTCGTCAAGCACATCAACCGGACCAAGA  475

Query  422  ACGCCATCCACAGCAGCATCGTCGACTTCTCCGGTAAGGGACCCGGCCACGAAGTGGAGA  481
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  476  ACGCCATCCACAGCAGCATCGTCGACTTCTCCGGTAAGGGACCCGGCCACGAAGTGGAGA  535

Query  482  TCGCGATGCAGTGGAATGCCGGCTATTCGGAGTCGGTGCATACCTTCGCCAACACCATCA  541
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  536  TCGCGATGCAGTGGAATGCCGGCTATTCGGAGTCGGTGCATACCTTCGCCAACACCATCA  595

Query  542  ACACCCACGAGGGTGGGACCCACGAAGAGGGGTTCCGCAGCGCGCTGACCTCGGTGGTGA  601
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  596  ACACCCACGAGGGTGGGACCCACGAAGAGGGGTTCCGCAGCGCGCTGACCTCGGTGGTGA  655

Query  602  ACAAGTACGCCAAGGACCGCAAACTGGTCAAGGAAAAGGACCCCAACCTCACCGGCGACG  661
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  656  ACAAGTACGCCAAGGACCGCAAACTGCTCAAGGAAAAGGACCCCAACCTCACCGGCGACG  715

Query  662  ACATCCGGGAAGGGTTGGCCGCGGTGATTTCGGTCAAGGTCAGCGAGCCGCAGTTCGAGG  721
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  716  ACATCCGGGAAGGGTTGGCCGCGGTGATTTCGGTCAAGGTCAGCGAGCCGCAGTTCGAGG  775

Query  722  GCCAGACCAAGACGAAACTGGGCAACACCGAGGTGAAGTCGTTCGTGCAGAAGGTGTGCA  781
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  776  GCCAGACCAAGACGAAACTGGGCAACACCGAGGTGAAGTCGTTCGTGCAGAAGGTGTGCA  835

Query  782  ACGAACAGCTCACCCATTGGTTCGAGGCCAACCCCGCTGACGCTAAAACCGTTGTCAACA  841
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  836  ACGAACAGCTCACCCATTGGTTCGAGGCCAACCCCGCTGACGCTAAAACCGTTGTCAACA  895

Query  842  AGGCGGTTTCATCGGCGCAAGCACGCATTGC  872
            |||||||||||||||||||||||||||||||
Sbjct  896  AGGCGGTTTCATCGGCGCAAGCACGCATTGC  926
```

c) 16S-23S rRNA ITS region sequencing

gi|146218732|emb|AM709725.1| *Mycobacterium kansasii* partial 16S rRNA gene, partial 23S rRNA gene and ITS1, isolate K7377-03
Length=404; Score = 628 bits (340), Expect = 4e-177; Identities = 340/340 (100%), Gaps = 0/340 (0%); Strand=Plus/Plus

```
Query  1    CTCCTTTCTAAGGAGCACCACGAAAAGCATCCCAACAAGTGGGGTGCAAGCCGTGAGGGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  55   CTCCTTTCTAAGGAGCACCACGAAAAGCATCCCAACAAGTGGGGTGCAAGCCGTGAGGGG  114

Query  61   TTCTCGTCTGTAGTGGACGAAAGCCGGGTGCACGACAACAAGCAAAGCCAGACACACTAT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  115  TTCTCGTCTGTAGTGGACGAAAGCCGGGTGCACGACAACAAGCAAAGCCAGACACACTAT  174

Query  121  TGGGTCCTGAGGCAACACTCGGGCTCTGTTCGAGAGTTGTCCCACCATCTTGGTGGTGGG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  175  TGGGTCCTGAGGCAACACTCGGGCTCTGTTCGAGAGTTGTCCCACCATCTTGGTGGTGGG  234

Query  181  GTGTGGTGTTTGAGAATTGGATAGTGGTTGCGAGCATCAAATGGATGCGTTGCCCTACGG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  235  GTGTGGTGTTTGAGAATTGGATAGTGGTTGCGAGCATCAAATGGATGCGTTGCCCTACGG  294

Query  241  GTAGCGTGTTCTTTTGTGCAATTTTATTCTTTGGTTTTTGTGTTTGTAAGTGTCTAAGGG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  295  GTAGCGTGTTCTTTTGTGCAATTTTATTCTTTGGTTTTTGTGTTTGTAAGTGTCTAAGGG  354

Query  301  CGCATGGTGGATGCCTTGGCATCGAGAGCCGATGAAGGAC  340
            ||||||||||||||||||||||||||||||||||||||||
Sbjct  355  CGCATGGTGGATGCCTTGGCATCGAGAGCCGATGAAGGAC  394
```

Appendix V: Macrorestriction analysis, using PFGE, of *M. kansasii* genomic DNA after *Xba*I digestion.



NB: Numbers above the lanes represent isolates and M denotes the molecular weight marker, PFG MidRange II (NEB). bp, base pair

Appendix VI: Culture Media and Reagents

A. Culture media

Luria-Bertani (LB) broth

10g tryptone powder

5g yeast extract

10g sodium chloride

Up to 1000ml distilled water; autoclave

Luria-Bertani (LB) agar

10g tryptone powder

5g yeast extract

10g sodium chloride

15g agar power

Up to 1000ml with distilled water, autoclave

SOC medium (100ml)

2g Tryptone

0.5g Yeast extract

1ml 1M Sodium chloride

0.25ml 1M Potassium chloride

1ml 2M Magnesium chloride, filter sterilised

1ml 2M Glucose, filter sterilised

Add tryptone, yeast extract, sodium chloride and potassium chloride to 97ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M magnesium chloride and 2M glucose, each to a final concentration of 20mM. Bring to 100ml with sterile distilled water. The final pH should be 7.0.

Middlebrook 7H9 broth (BD-Difco Laboratories, Sparks, MD, USA)

4.7g Middlebrook 7H9 broth base

2ml glycerol

0.05% Tween

Up to 900ml distilled water

100ml OADC supplement added after autoclaving

Middlebrook 7H10 (BD-Difco Laboratories, Sparks, MD, USA)

19g Middlebrook 7H10 agar powder

2ml glycerol

Up to 900ml distilled water

100ml OADC supplement added after autoclaving

Dubos medium (BD-Difco Laboratories, Sparks, MD, USA)

1.3g Dubos broth base

30ml glycerol

70ml distilled water

(For preparing freeze culture stock: equal volume of medium and culture)

B. Reagents

TE-Triton X-100

100mM Tris, 1mM EDTA, pH 8.0

1% Triton X-100

ESP solution

0.5M EDTA, pH 8.0

1% Lauroyl sarcosine

Autoclaved and stored at room temperature

Proteinase K weighed and added at a concentration of 1-2mgml before use

TE buffer (pH 8.0)

10mM Tris HCl, pH 8.0

1mM EDTA

TBE (x1)

89 mM Tris

89 mM Boric acid

2mM EDTA

Spheroplasting Buffer (plus Tween 80) – autoclaved

20mM Citrate phosphate buffer (pH 5.6)

50mM EDTA

0.1% Tween 80

(200mM citrate phosphate buffer stock made by mixing 42ml of citric acid [0.2M], followed by 58ml sodium hydrogen phosphate [0.5M])

Ethidium bromide solution

10mg/ml in distilled water.

Stored in a dark bottle at $4^{o}C$

Lysis solution

TE, pH 8.0

Autoclaved and stored at room temperature

Lysozyme weighed and added at concentrations of 1-2mg/ml before use

Ringer's solution

8.6g sodium chloride

0.3g potassium chloride

0.33g calcium chloride

Up to a 1000ml of distilled water.

5M Sodium chloride

29.2g Sodium chloride in 100ml distilled water

Autoclave.

CTAB/Sodium chloride

Dissolve 4.1g sodium chloride in 80ml distilled water. While stirring add 10g CTAB (N-acetyl-N,N,N,-trimethylammonium bromide). If necessary, heat to 65^{o}C. Adjust volume to 100ml with distilled water and store at room temperature.

0.5M EDTA

186.12g EDTA

Up to 1000 distilled water

IPTG Stock Solution (0.1M)

1.2g IPTG

Add water to 50ml final volume. Filter sterilise and store at 4^{o}C.

X-Gal (2ml)

100mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

Dissolve in 2ml N,N'-dimethylformamide. Cover with foil and store at -20^{o}C.

Maleic acid buffer (pH 7.5)

0.1M Maleic acid

0.15M Sodium chloride

20X SSC Buffer

3M Sodium chloride

0.3M Sodium citrate

Pre-hybridization Buffer

5X SSC buffer

0.1% N-lauroyl sarcosine

1% Blocking reagent

0.02% Sodium dodecyl sulphate

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REFERENCES

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